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CONTENTS

	PAGE
THE SIGNIFICANCE OF HEMOGLOBIN. *Joseph Barcroft*	329
INTESTINAL AUTOINTOXICATION. *Walter C. Alvarez*	352
SULFUR METABOLISM. *Howard B. Lewis*	394
THE NUTRITIVE VALUE OF PROTEINS. *H. H. Mitchell*	424
THE MECHANISM OF VOMITING. *Robert A. Hatcher*	479
PROTEINS AND THE DONNAN EQUILIBRIUM. *David I. Hitchcock*	505

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PHYSIOLOGICAL REVIEWS

VOL. IV JULY, 1924 No. 3

THE SIGNIFICANCE OF HEMOGLOBIN

JOSEPH BARCROFT[1]

Physiological Laboratory, Cambridge, England

The title may be held to include some statement of the utility of hemoglobin to the organism and of the degree to which hemoglobin is adapted to the uses to which it is put. If nature adapts a certain chemical substance to a certain end, it might be supposed that the material in question is ideal for the purpose. Surely there can be no such presumption—an easier thesis to defend would be that no superior chemical compound was available, that everything had been tried and that in one way or other, during the course of evolution, the less desirable had always been discarded and the most desirable had everywhere been discovered. Even this lesser proposition would be difficult of proof, yet it falls far short of a statement that nature has always found or improvised something which never falls short of her demands. Had she been able to do so I suppose that progress would have run a much more rapid course than it has done; but also physiology would have been much duller. I like to think of organic nature as one who puts forth vast programmes on small means, occupied always in the manufacture of unlimited bricks with but too little straw. I count it as no insult to her to find out that her resources are deficient, to discover where this or that substance fails of its object, to be forced to the conclusion that this or that material is in the abstract, rather unsuitable but that being the best obtainable nature has resorted to a dozen contrivances in order to obtain the very maximum of which this material is capable.

I would like to treat of hemoglobin along these lines, to give an account of its properties, to state where it first appears in the scheme of nature, to explain the precise purpose which it is there serving, and

[1] Doctor Barcroft has consented to prepare a second article on the functional significance of hemoglobin in the lower forms of animal life, which will be published in 1925.—Editor.

trace the successive devices which are invoked in order to make it meet the ever increasing demands which are put upon it through the ascent of the animal kingdom. Such a scheme is impossible. It is impossible to say where hemoglobin first appears, and even if we could do so it would not be easy to state precisely what it is doing there. Likely enough it would be difficult to draw any precise line between its function and that of some other pigment—its next door neighbor. Or it might have no function, that would be the most significant discovery of all for then we would have hit upon its accidental occurrence. We would know indeed that we had discovered a possible starting point for we could say: Here hemoglobin is found apparently as an accident, it serves no end, it apparently exists as a chance, but this may be the chance of which evolution has taken advantage.

The opposite procedure must therefore be adopted. In the highest animals the significance of hemoglobin, or at least its principal significance, is fairly evident. How far back can we trace the pigment without losing our certainty of its function?

In man its significance is as a carrier of oxygen; with that function is associated another, namely, that of facilitating the transport of carbonic acid. These functions have been so much canvassed within the last decade (1) (2) (3) (4) (5) (6) (7) (8) that it would be superfluous here to treat of them at great length, but a quantitative statement of the significance of hemoglobin may not be out of place. The oxygen which a man requires depends upon the work he performs. To insure his getting the necessary supply his blood has an oxygen capacity of about a liter. A liter of plasma saturated with alveolar air takes up about 3 cc. of oxygen, therefore in order that the vascular system should hold a liter of oxygen in solution it must needs contain 300 or more liters of fluid—four times the weight of the man himself. Even after allowance has been made for the fact that plasma could be made to circulate much more rapidly than blood and considerations of the kind, the burden of the vascular system would be unbearable. It would immobilize the individual. The warm-blooded creation owes its existence, or at all events, its activity to hemoglobin.

It is not merely that in a general sense a plentiful supply of hemoglobin has been meted out to those forms of life whose need for oxygen would otherwise far outrun their capacity for obtaining it, but even among warm-blooded animals the average hemoglobin value of the blood varies from species to species. In two such closely allied species as the dog and the cat a great disparity exists, the former having about

half again as much hemoglobin as the latter. Nor is this all, for even in the same species the quantity of hemoglobin is regulated apparently in order to adjust the demand for oxygen to the supply. The dwellers in the high land which separates the two Andean Cordilleras possess blood which has a high hemoglobin content, as the following figures show. Of twelve native residents at between 14 and 15,000 feet in the Cerro de Pasco region (9):

	HEMOGLOBIN VALUE (NORMAL SEA LEVEL = 100)				
	150	140 –149	130– 139	130 –129	Below 120
Number of cases	1	3	4	4	0

Another instance which is essentially of the same sort, namely, one in which an excessive hemoglobin value in the blood was coupled with a deficient mechanism for oxygen supply to the tissues of the body, was that of a very interesting case recently described before the British Physiological Society by Haldane and his colleagues. It was one of congenital foramen ovale, so that a portion of the blood which reached the right side of the heart was short circuited and mixed on the left side with the highly oxygenated blood which came from the lung. The resulting arterial blood was 86 per cent saturated therefore of the same order as in dwellers at Cerro de Pasco in whom an excessive hemoglobin content was observed Similar cases are described by Campbell, Hunt and Poulton (10).

When one passes in the other direction from the warm-blooded to the cold-blood vertebrates, the hemoglobin value of the blood sinks. Quite a number of factors tend to make the demand for oxygen more nearly commensurate with the supply which could be maintained by a blood devoid of pigment. In the first place the demand is less, thus a frog requires about 0.05 cc. of oxygen per gram per hour (11), whilst a mouse of the same weight would require upwards of 5 cc.—a hundred times as much. Secondly, at the lower temperature of the frog, oxygen is considerably more soluble in the plasma and thirdly, owing to the low pressure both of aqueous vapor and carbonic acid in the lung of the frog the blood is there exposed to a considerably higher oxygen pressure than it is in the human lung, possibly to about 130 mm. Taking these last two factors together, each cubic centimeter of plasma which leaves the frog's lung would contain about twice as much oxygen as that which leaves the lung of the mouse. Looking at the posi-

tion both from the view of demand and from the view of supply, the frog without hemoglobin would thus be about 200 times better off than the mouse. That it is not simply a question of phylogeny, hemoglobin looming larger as the vertebrate phylum is ascended, is shown by the consideration of avian blood. Birds have blood rich in hemoglobin, for precisely the same reason as have mammals, namely, that their activities would be impossible without an intense metabolism, and an intense metabolism is impossible in an organism of any size without a copious supply of hemoglobin in the blood. Insects are of course capable of very rapid movements and presumably their muscles are the seat of rapid oxidation. The oxygen is conveyed by diffusion along tracheal tubes of very narrow dimensions. Diffusion along such tubes is however a slow process and for this reason, as J. B. S. Haldane has pointed out, the active insect must ever remain minute, the terminations of its tracheal tubes can never be far removed from their external openings.

For this reason I take it that an insect can never be capable of a high degree of intelligence—this in spite of all that has been written about the ant—for a high degree of intelligence demands a brain too complicated and massive to be served by a tracheal system.

Nature has only found it possible to combine bulk and activity in the mammal by the provision of an intensive system of chemical transport for the gases involved in respiration. In hemoglobin, and in hemoglobin alone, she would appear to have found a substance whose qualities are sufficiently adapted to the demands put upon it. The degree to which it is made use of in the vertebrate phylum is a matter not of the phylogeny but of the physiology of the organism.

The adaptability of hemoglobin. Let us proceed to consider the extent to which hemoglobin in its rôle as a transporter of oxygen meets the needs of the warm-blooded animal. *1.* It should be capable of carrying a sufficient supply of the gas. *2.* In the capillaries of the lung and of the tissues it should acquire and part with its oxygen at rates which are sufficiently rapid and not very unequal. Concerning the first point enough has been said in the preceding paragraphs. The second may be dealt with rapidly for Hartridge and Roughton have shown in the very beautiful researches which they have published recently (12), that the velocities of the chemical processes of oxidation and reduction of hemoglobin are very rapid as compared with the physical processes involved. The actual time required for the chemical process of disunion between hemoglobin and oxygen is to be measured

in thousands of a second and that of union between the two is even more rapid.

Roughton (13) has shown also that the pressure of oxygen in the blood over all parts of a cross section of a capillary may be regarded as sensibly the same. The rate at which oxygen will enter the capillary then depends upon the rate of diffusion of oxygen from an atmosphere which (as a first approximation) may be considered to contain 100 mm. of that gas into a solution of hemoglobin which at any point has an oxygen pressure which is regulated by its oxygen dissociation curve. Similarly let us consider the rate at which oxygen would leave the capillary. This again varies with the difference between the pressure in the capillary, which also is determined by the oxygen dissociation curve. Let us assume the optimum conditions for the passage of oxygen from the capillary, namely, an oxygen pressure outside it of *nil*. Let us suppose then that we have a standard capillary (see fig. 1 V), with the hemoglobin running through it at a uniform speed, that when the hemoglobin enters the capillary 95 per cent of it is oxyhemoglobin and 5 per cent is reduced hemoglobin and that it traverses the capillary till 95 per cent is reduced and the remaining 5 per cent is oxidized, there being no oxygen in the atmosphere outside the capillary. The length of time which any small quantity of hemoglobin will take to lose its oxygen will vary inversely as the difference between the oxygen pressure in the fluid and that outside. For instance, if the oxygen pressure is 2 mm. in the fluid and nil outside, then $\frac{1}{2}$, i.e., 0.5, may be taken as a measure of the time required for unit quantity of oxygen to leave the capillary at the spot when the pressure is 2 mm. From the dissociation curve (fig. 1, I) we can obtain the pressure which corresponds to each percentage saturation along the curve and we may plot the reciprocals spacing them parallel to the abscissa at the point corresponding to the percentage saturation to which they belong. See figure 1, curves I to IV. Curve I represents the equilibrium between oxygen, oxyhemoglobin and reduced hemoglobin at the temperature of 16°C. The black area to the left represents the integration of the reciprocals of the pressures of oxygen which corresponds to each percentage of saturation and therefore in the units adopted the area is a measure of the whole time that would be necessary in order to reduce the blood from 95 to 5 per cent saturation. Still considering the conditions represented in figure 1 V to figure 1 I, let us pass to the rate at which the hemoglobin can be oxidised. We suppose a mixture of 5 per cent oxy- and 95 per cent reduced hemoglobin to enter a capillary around which

the oxygen pressure is 100 mm. The pressure of oxygen in the fluid is 0.02 mm. that subtracted from 100 mm. is 99.98. The reciprocal of this is approximately 0.01 and is therefore no thicker than the line which bounds the dissociation curve. The relative times required for the oxidation and reduction are of different orders, the black area to the left, which represents the time for reduction being about six thou-

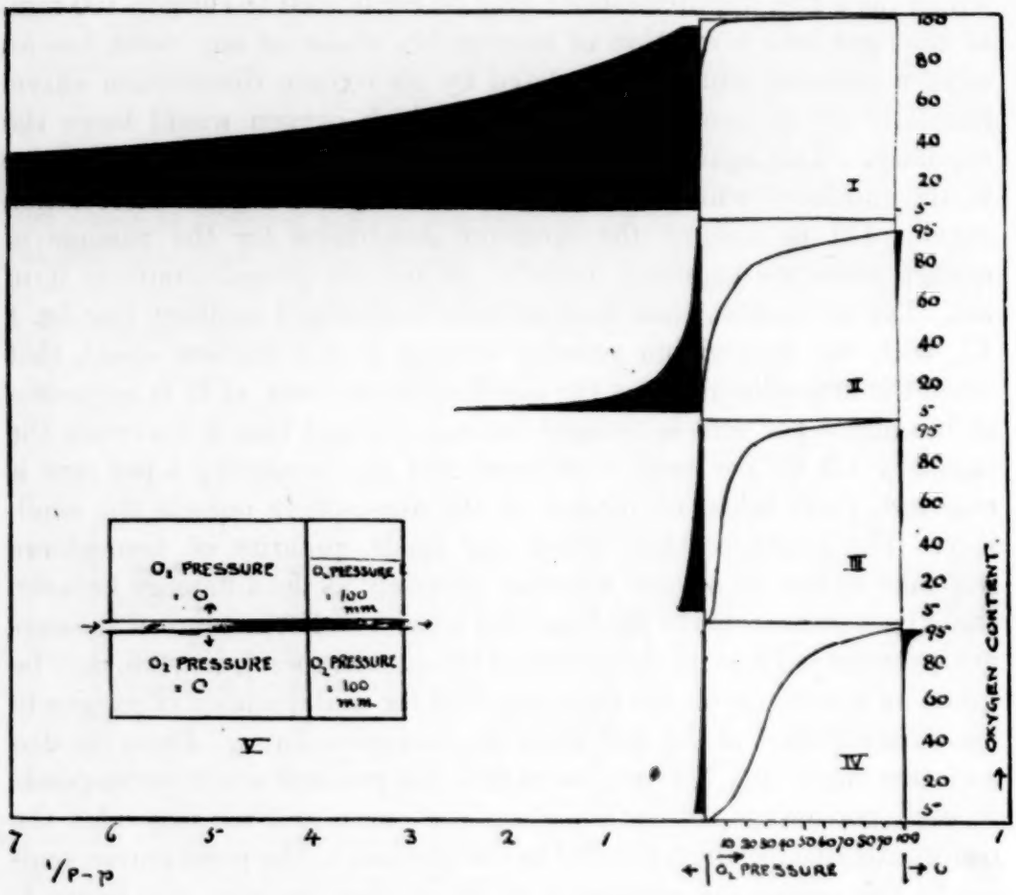


Fig. 1

sand times as great as that on the left which represents the time for oxidation expressed in the same units. Given a blood consisting of a solution of hemoglobin in water, if one second were required for its oxidation in the lung capillary an hour and a half would not suffice for its reduction in the tissue capillary. Or one's imagination may run riot in another direction. If a capillary 1 mm. long sufficed for the oxidation, one about 20 feet long would be required for the reduction, granting that they were of the same caliber and permeability and that

a stream of fluid ran through both at the same velocity. Imagine what the human frame would be like if for each capillary of a millimeter of length in the lung there was one of 20 feet long in the tissues. It would indeed be a *rete mirabile.*

Let us trace step by step the means which nature has adopted to reduce this disparity. It does not greatly matter in what order they are considered. First then the temperature of the reaction has been raised to 37°C. The equilibrium is represented in curve II. The relative times required for oxidation and reduction are depicted as before. They are here about as 25 to 1. This is very different from 6000 to 1, nevertheless it is sufficient to remind one of what Haldane (14) said about Hüfner's curve—which as a matter of fact was practically identical with curve II. "A man would die on the spot of asphyxia if the oxygen dissociation curve of his blood were suddenly altered so as to assume the form which Hüfner supposed it to have in the living body."

The next change appears in curve III, which is the equilibrium curve between oxygen and blood at 37°C., but in the absence of CO_2. The essential departure is from the hyperbolic to the S-shaped form. The advantage of this type of curve expressed graphically is that, down almost the whole length of the curve, the gap between it and the axis is kept open and therefore the reciprocal of that gap is correspondingly reduced. The relative sizes of the black areas related to curve III are not very different from those related to curve II. The advantage of curve III consists in its capability of further development. Curve II leads to nothing. At the top of the oxidation area a lengthy spike is commencing to appear at the top of the area, this means to say that 95 per cent saturation is scarcely possible of attainment with 100 mm. oxygen pressure, however long the time given. Consider now the possible methods for the further improvement of curve II. Of these there are a couple, namely, a further rise of temperature and an adjustment of the hydrogen ion concentration. They both have the same effect, namely, to increase in the same ratio the pressure at each percentage saturation. This, it is true, would reduce the reduction area somewhat especially at the top. But the curve would never reach the top. The main effect would be to increase the oxidation area, to accentuate the spike to which attention has just been called, to bring it lower down. The areas would become more equal, but the equality would be gained rather by increasing the inefficiency of oxidation than by decreasing that of reduction.

Curve III passes by a suitable adjustment of the hydrogen ion concentration to curve IV—that of arterial blood as it is in the body. The effect upon the oxidation and reduction areas is very marked. It makes them nearly equal in extent, as nearly as possible uniform in breadth from top to bottom and their combined areas the smallest possible, representing graphically the facts that the oxidation and reduction times of blood are of the same order, that the rates at which the two processes take place are as nearly uniform as may be over their whole course and that taken together they are as short as possible.

Having thus indicated the pains at which nature has been to make the best of hemoglobin, the fact must be emphasized that although hemoglobin seems far from being ideal, it is yet probably the best substance available. In particular two points should be emphasized. One is connected with each of the factors to which allusion has just been made, namely, the temperature and the doubly inflected curve. Hemoglobin is remarkable apparently in that the equilibrium constant of its reaction with oxygen has a temperature coefficient of about 4. This figure is so high as to have been received with considerable scepticism by many and to have evoked a certain amount of adverse comment. I therefore use the word "apparently" in order to make it clear that I have quite an open mind and that I should welcome any revision of it. So far, however, the evidence which has cropped up since Hill and I (15) published it has been in its support. Brown and Hill (16) have confirmed it on human blood and Hartridge and Roughton (12) have, in experiments which I think they regard as being too few to be final, obtained substantially the same figure for dilute hemoglobin solutions at pH = 7.5 per cent. So far as their experiments go they arrive at the result that the velocity constant of one phase of the reaction has a temperature cofficient of 1, being apparently unaffected by rise or fall of temperature, whilst the other phase has a temperature coefficient of 4, which figure therefore is also that of the coefficient of the equilibrium constant.

The mechanism of the double inflection. The second significant thing about hemoglobin is that the curve which represents its equilibrium with oxygen should be capable of diversion from a hyperbolic to a doubly inflected curve.

The mechanism responsible for this change must at present be treated with great caution. A while ago I had supposed that any solution of hemoglobin, were it pure enough, would give a hyperbolic oxygen dissociation curve, whilst any solution which contained salts

or CO_2 would give an S-shaped curve. This simple view of the matter clearly falls far short of the facts as they are now known. The factor which appears to have been left out of consideration is the effect of the concentration of hemoglobin on the form of the curve. It is impossible at present to make any final statement, but the following is a summary of the facts at present available, worked as nearly as may be into a complete scheme.

1. If the solution of hemoglobin is sufficiently dilute, say such as may be obtained by the dilution of 1 cc. of blood in 70 or 100 cc. of distilled water, and is therefore about 1/500, the oxygen dissociation is hyperbolic whether the curve does or does not contain salts; whether it is neutral acid or alkaline (12)—a fact which appears to be in direct and interesting conflict with what might be predicted from a study of the osmotic pressures of hemoglobin solutions (17).

2. If the solution is stronger, say of the order of concentration used by Roberts and myself (18), the curve is never quite hyperbolic, but under certain circumstances is, as we found, nearly so. The circumstances are as follows:

a. That it should be free from salts.

b. That it should be removed a little on one side or other from the iso-electric point. A salt free solution of hemoglobin at the iso-electric point gives an S-shaped curve, and the range of hydrogen ion concentrations over which the curve maintains its S-shaped character is greatly extended by the addition of sodium chloride.

3. In the strongest concentrations available as found by Adolph and Ferry (20) (and we at Cambridge, England, have frequently confirmed their observations) it is S-shaped under all circumstances.

The concentration of the hemoglobin reaches its maximum in the red blood corpuscle, and therefore we may at this stage consider the next device of nature in the handling of hemoglobin, that of placing it in a special cell which is carried round in the circulation. This matter may be dealt with very briefly since already I have treated of it at some length in a lecture delivered to and published by the Harvey Society (21). Briefly, it amounts to this: a solution of hemoglobin in the concentration in which it occurs in blood, possesses certain physical properties which would make its occurrence in the circulating fluid extremely deleterious to the organism:

1. It would have an osmotic pressure of more than 100 mm.
2. It would add materially to the viscosity of the blood.

3. It would be propelled according to the laws of stream line motion and therefore in effect only a fraction would circulate whilst the remainder would more or less stagnate along the capillary wall, not only being itself wasted but increasing the thickness of material through which the oxygen must perforce diffuse.

4. Unless the capillary wall were less permeable than it is in the mammalia and therefore less efficient, the hemoglobin would diffuse out into the tissue spaces.

All these disadvantages are obviated by the enclosure of the pigment in cells commensurate in diameter with the bore of the capillary. In addition much is gained on the chemical side, since the intracorpuscular atmosphere may be adjusted so as to place the hemoglobin in the environment in which it is most efficient as a transporter of oxygen and carbonic acid between the tissues and the lungs. That atmosphere need not necessarily be, and indeed is not, the atmosphere of the plasma.

To take one point only, so far as we can see the conditions which decide the optimum hydrogen ion concentration are first, that the ratio of the rates of oxidation and reduction of blood should be most nicely balanced, and secondly, that alterations of hydrogen ion concentration which take place during pulmonary and tissue respiration should have their maximum effect upon the exchange of gases. Both these points seem to be gained by placing the hemoglobin in a medium whose hydrogen ion concentration is slightly greater than that of the plasma, as the inside of the corpuscle has been shown to be by the numerous researches of Milroy (22), Campbell and Poulton (23), Conway and Stephen (26), Warburg (27) and Taylor (29).

The physiological significance of the corpuscle as providing an independent medium for the hemoglobin may be illustrated by two, out of many examples which might be cited. The first has to do with the adaptation of hemoglobin to the different needs of different species, the second to the varying circumstances in which a single individual may find himself.

The first I quote from Krogh (30) who drew attention to the different physiological conditions under which different species of fish live; some in the mud where the tension of oxygen is very low, others near the surface of the sea and of rivers where the water contains a considerable quantity of oxygen in solution.

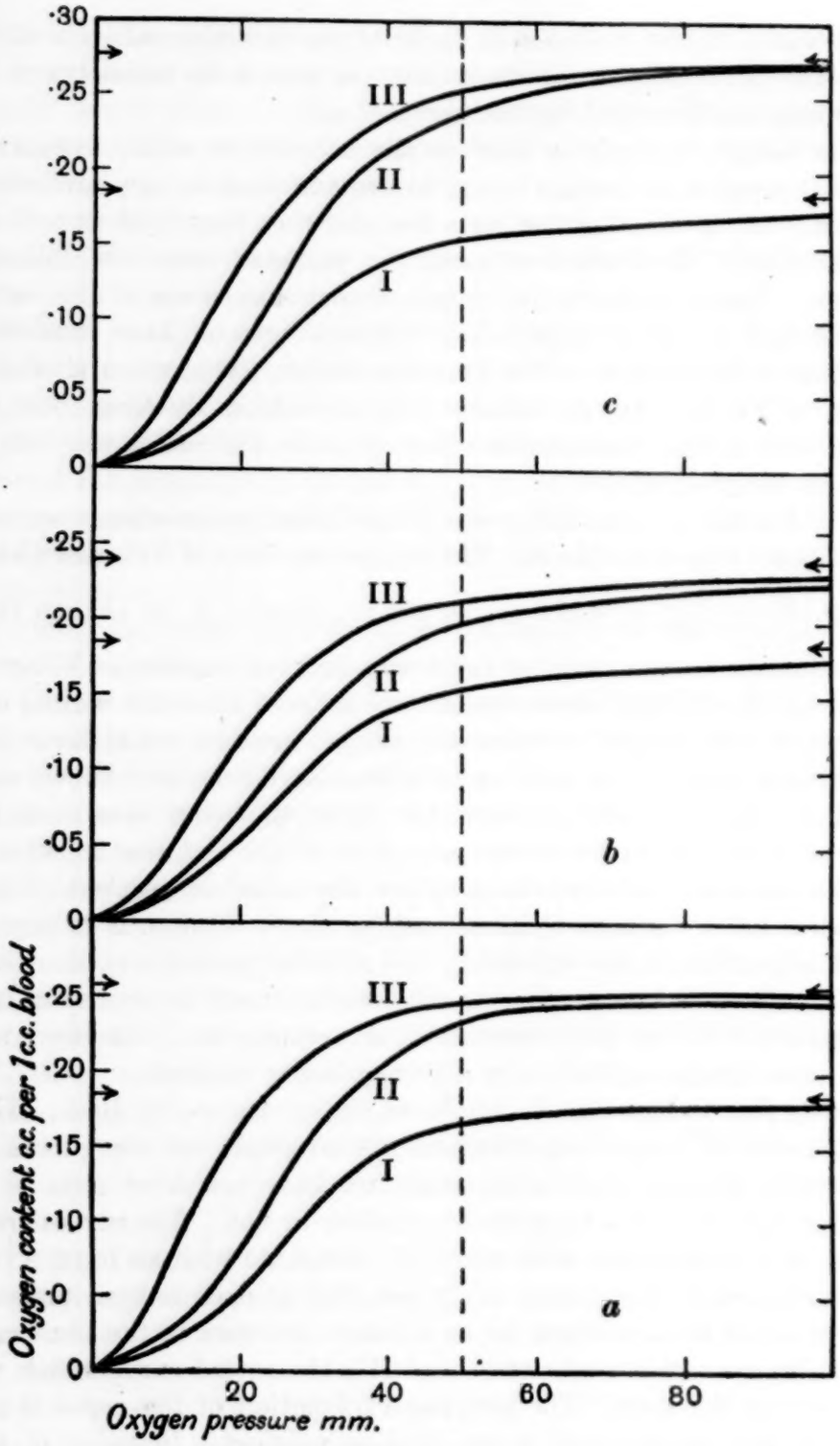
As Krogh saw, either the hemoglobins of these fish are not the same or being the same they are placed in different environments. Since

there was no direct evidence in favor of the first alternative he attributed the differences in the dissociation or curves to variations in the environments furnished by the corpuscles.

The second example is that of the adaptation which appears to make it possible for human beings to live and work in very rare atmospheres. Let us consider the facts first and then pass to their probable explanation. The facts can easily be gathered from the following figures. Figure 2a shows the oxygen dissociation curves of I, a normal person, and III, of a man called Villareal, one of those studied by the 1921–2 Expedition to the Peruvian Andes, living at an altitude of about 14,200 feet, he, in common with all natives dwelling there had blood with a high hemoglobin value (9). In his case it was 148 per cent of the normal.

Curve I is that of a normal person whose blood has an oxygen capacity of 0.185 cc. O_2 per cc. blood. The oxygen capacity of Villareal's blood was $0.185 \times \frac{148}{100} = 0.274$ cc. O_2 per cc. blood. If no change other than a mere concentration of corpuscles had taken place in Villareal's blood his dissociation curve would have differed from the normal only in that at any oxygen pressure the oxygen content would have been 48 per cent greater, the curve would then have been that shown as II in figure 2a. Actually however his curve was that shown as III. Curves II and III arrive at the same level in the end, that is, when an oxygen pressure sufficient to saturate the blood is reached, but at pressures below 50 mm. O_2 (and nothing above 50 mm. is of any account since that is approximately the alveolar pressure at these altitudes) Villareal's blood takes up considerably more oxygen than is to be accounted for by mere increase of the corpuscles. The curve suggests that the hemoglobin is in a more alkaline medium.

Let us pass to figure 2b, which shows similar curves for Bock. Here the increase in oxygen capacity was not so great, but the curves are instructive because concerning Bock we have sea level data as to whether his blood was abnormally alkaline or not. The curves are as before, I, a normal sea level curve; II, the same with an oxygen content increased to the extent of 28 per cent at each oxygen pressure, such as could be accounted for by a simple increase in the number of corpuscles per cubic centimeter, and III, the actual curve which was obtained on the spot. The simplest explanation of the curve is also that the hemoglobin was in an alkaline medium. In point of fact the hydrogen ion concentrations of his blood as measured by the CO_2 bicarbonate ratio were at


·30
·25
·20
·15
·10
·05
0
III
II
I
c
·25
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·15
·10
·05
0
III
II
I
b
·25
·20
·15
·10
·0
0
III
II
I
a
20
40
60
80
Oxygen content c.c. per 1 c.c. blood
Oxygen pressure mm.

Fig. 2

Sea level pH = 7.34
Cerro de Pasco = 7.35
7.35

and the whole evidence gleaned from our party showed no change in the reaction of the plasma commensurate with that which would be required to explain the difference between curves II and III on the basis of increased alkalinity. Is it possible then to alter the reaction of the inside of the corpuscle without producing the same alteration in the plasma? Cecil Murray (31) suggested a way which this would be accomplished and in figure 2c are curves obtained by Doctor Uyeno and myself (32) which indicate that just such changes as take place at high altitudes may be obtained in drawn blood by taking advantage of the laws which govern the interchange of acids and bases as between the corpuscles and the plasma. If from blood containing 40 mm. CO_2 most of the carbonic acid be shaken the chloride will migrate in part from the corpuscles into the plasma, if then 10 cc. of the blood be centrifuged and 2 cc. of the plasma withdrawn, more than $\frac{1}{5}$ of the chlorides will have been taken away; if lastly, the blood be equilibrated with the appropriate pressure of CO_2, say 25 mm. (that being about the alveolar CO_2 pressure at Cerro), the plasma will be no less acid than would the original plasma have been if equilibrated with CO_2 since it contains additional chlorides and the corpuscles will be more alkaline since they contain both less chlorides and less CO_2. Figure 2c, curve I, is my normal blood, II is the same concentrated without loss of CO_2 and curve III is treated in the way described above. In it the alkalinity of the corpuscles is reduced but not that of the plasma.

Before leaving the consideration of figure 2 we may refer to the conditions at the highest known altitude at which the alveolar oxygen pressure is calculated to be 23 mm. If no further adaptation took place than what is known in Villareal's blood, the quantity of oxygen in the arterial blood would be about the same at the summit as is normal at sea level, namely, 0.18 cc. per cubic centimeter of blood.

The significance of hemoglobin as a buffer. Hitherto we have considered the significance of hemoglobin as a carrier of oxygen, there are several other functions which it may perform, these in a sense are subsidiary, at least in the mammalia. It may for instance act as a catalyst (33), (34), it may act as a vehicle for the secretion of iron, it may have an importance as a pigment. Probably these functions become of more practical interest as we descend the animal kingdom and it is open to argument whether or not they eclipse the oxygen-

transporting function in some of the lower forms of life; but the function of hemoglobin as a buffer seems essentially best developed in those creatures whose blood contains the pigment in the highest concentration. Such are the mammals and the birds. These are of course the forms which are most capable of sustained activity on the great scale. That hemoglobin is a buffer need not be argued here. It is its significance which must be discussed. The important point is that by buffering the blood, hemoglobin incidentally buffers the tissues. A certain quantity of CO_2 is thrown into the blood, this as we believe acquires sodium or potassium from the hemoglobin and therefore produces a relatively small rise of carbonic acid pressure in the blood; and because the carbonic acid potential is kept down in the blood its rise is relatively small in those tissues where the carbonic acid is being produced: the hydrogen ion concentration of these tissues therefore undergoes relatively little change.

The above statement, which merely assumes that hemoglobin acts as a weak acid, greatly underestimates its efficiency as a buffer. In the animal economy, however, it usually happens that at the precise moment when carbonic acid is entering the blood, oxygen is leaving it. That is to say, a certain amount of hemoglobin is being transformed from the oxy- to the reduced condition. This oxy-hemoglobin would appear to be about 70 times as strong an acid as reduced hemoglobin and indeed not to be very different in strength from carbonic acid itself (36). The effect of the reduction of the blood is to loosen the grip which the hemoglobin has of the base and therefore to transfer more base to the CO_2 than would otherwise be the case.

The buffering of the blood may be treated very simply by the application of the formula (7) true only in the physiological region (51).

$$vCO_2 = b \times cH \times 10^{\beta} + c$$

vCO_2 being the volume of CO_2 in the blood, cH being the hydrogen ion concentration (*not* its logarithm), b and c being constants. Of these two constants b is clearly the volume of CO_2 which the blood acquires for an increase of $1 \times cH \times 10^{\beta}$, in short the degree to which the blood is buffered, (10) while c is the quantity of CO_2 left in the blood when all involved in the hemoglobin-bicarbonate buffer system is shaken out. In point of fact, at the lowest part of the curve the equation is only an approximation, this residual sodium carbonate breaks down giving free alkali, nevertheless it is instructive to treat it as reality, because it may be regarded as the scaffold on which the curve

is built. The curve essentially is influenced by two factors *1*, *b* the buffering which increases or decreases with the quantity of hemoglobin present; *2*, *c* which increases if alkali is added to the blood and which decreases if fixed acid is added.

The following examples of the graphic application of this equation may be cited. Meakins arrived at Cerro de Pasco (altitude 14,200 feet) on December 24, 1921 and on December 26 his CO_2 dissociation

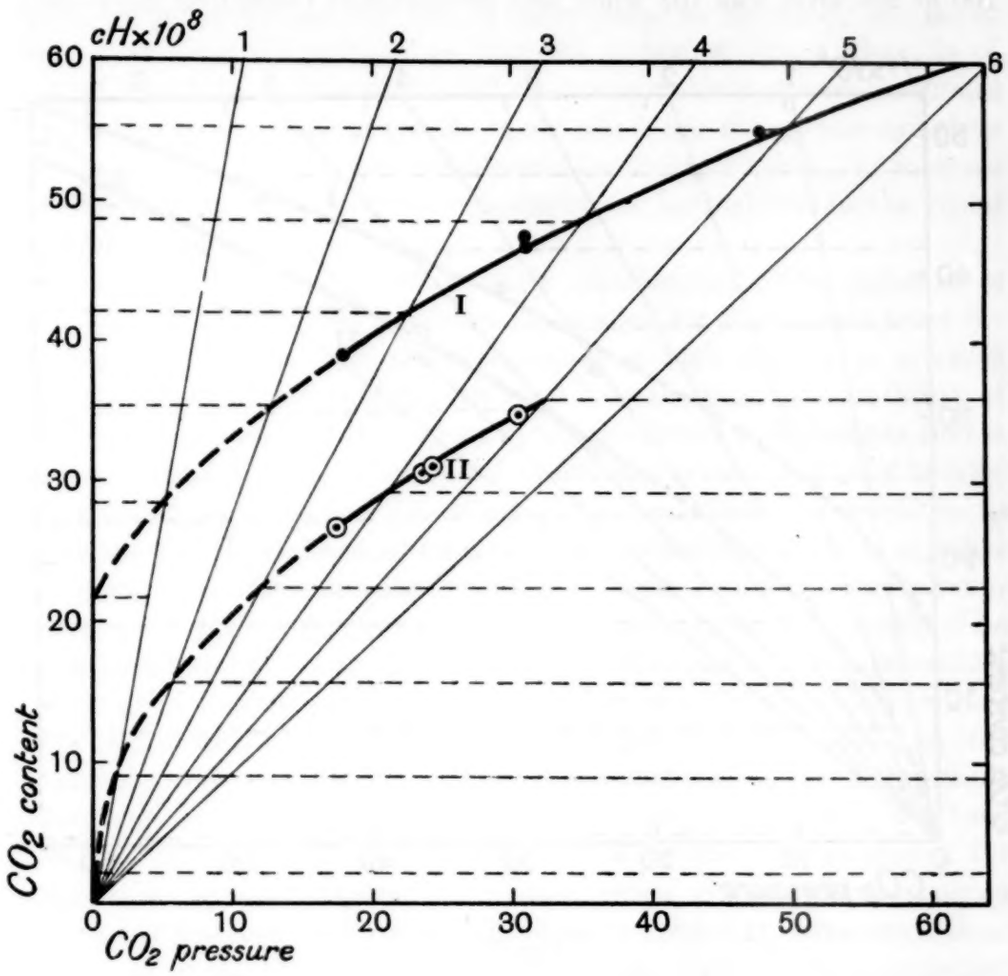

Fig. 3a

curve was determined. Figure 3a shows the observed points (with the exception of one which was quite out of the picture) through the point is drawn the line (curve I) corresponding to an alkali value *c* of 2.5 and a buffer value of *b* of 6.75. This may be compared with his normal (curve II) which has sensibly the same value for *b* but which has a much greater value for *c* of 21.5. The deduction would be that

an amount of alkali which would unite with 19 volumes of carbonic acid had been secreted from the blood as the result of this ascent, but that in so short a time there has been relatively little concentration of the blood, as indeed was borne out by the fact that Meakins' hemoglobinometer reading was only 106 (his normal tending to be rather below 100). With Meakins' blood may be contrasted that of Harrop.

Harrop's hemoglobin value which had also probably been below 100 at sea level was 102 when first estimated at Cerro four days after

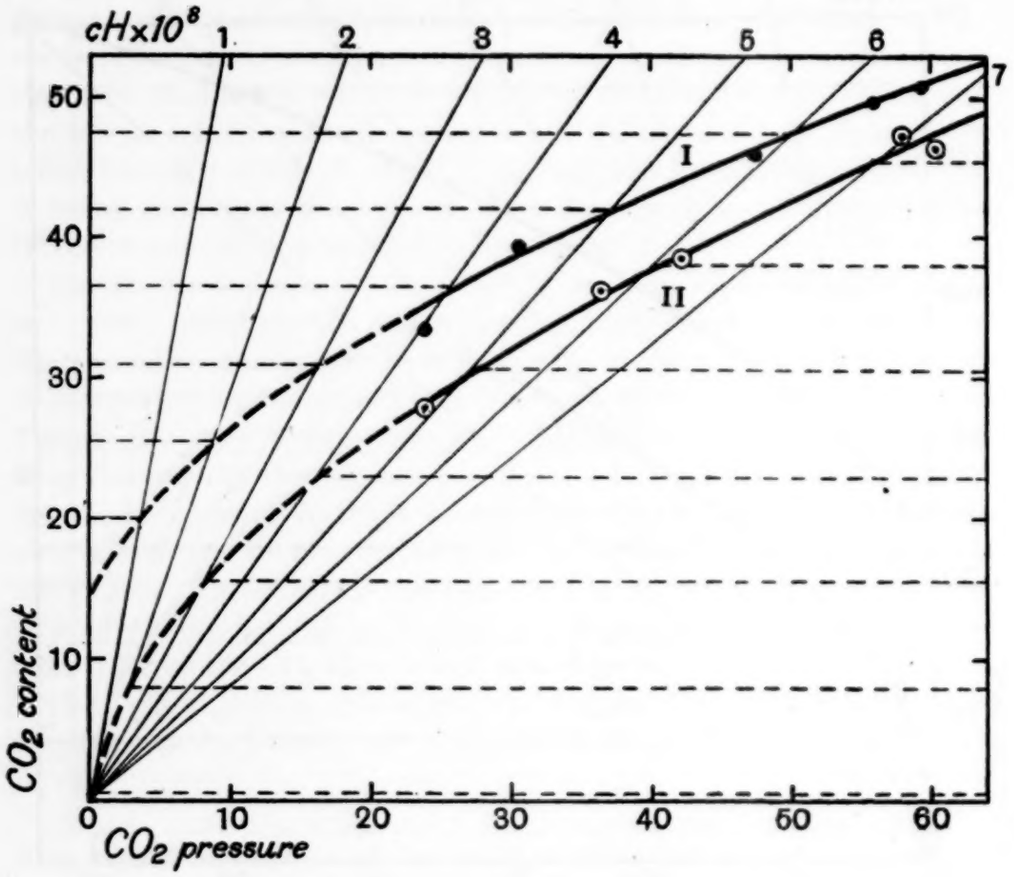

Fig. 3b

his arrival. On January 6, i.e., after about a fortnight's residence at Cerro, it was 118. It may be said confidently to have risen 18 per cent —probably a little more. Figure 3b shows the comparison of his CO_2 dissociation curves, at Lima (I) and on January 6 at Cerro (II), the former approximates to an equation

$$CO_2 = 5.5\ cH + 14.5$$

the latter to:

$$CO_2 = 7.5\ cH - 6$$

The buffer value of the blood is increased from 5.5 to 7.5 while the value for c becomes a minus quantity. The reversal of sign is partly due to the demand which the increased hemoglobin makes upon the sodium and partly to the excretion of alkali, there is therefore less available alkali in the blood than the hemoglobin is capable of uniting with. The percentage increase in buffer value as shown by the curve is greater than the percentage increase in hemoglobin. There is of course a certain error in each, and moreover a slight correction should be applied to the isopleth for the blood which has the greater quantity of hemoglobin, but the correspondence is sufficient to show the increase of buffering involved in the accumulation of hemoglobin in the blood at high altitudes.

Granting the facts, what is their significance? The question is not easy to answer, yet it cannot be ignored for it suggests some interesting reflections. Of course the effect of high altitude is to cause oxygen want, the effect of oxygen want is to increase the irritability of the respiratory center, the effect of this increased irritability is that a given increment of C_H in the blood produces a greater degree of breathlessness; so far so good. The effect of increased buffering would be to mitigate the breathlessness because a given addition of CO_2 or other acid would in the more highly buffered blood produce a lesser increment of C_H than formerly. Is this an advantage or otherwise? The breathlessness itself may be regarded as, and in fact is, a compensation. A too great buffering of the blood would seem to be an undoing of the good work already accomplished. We might leave the matter in this state of doubt, but for some other considerations. It is not credible that the whole mechanism of compensation to anoxemia has been devised for the facilitation of aeronautics or the encouragement for the ascent of Mt. Everest. No considerable portion of the human race or of the ancestral races which have preceded it, have owed their existence to the altitude to which they could attain. We may regard the mechanism as purely accidental; but if we do not, its raison d'être must be sought elsewhere at lower altitudes than those of the Andes and the Himalayas. We naturally turn to the survival of disease. Under what circumstances of disease does concentration of corpuscles take place? There is surgical shock, a condition which by the increased blood count cannot be attributed primarily to anoxemia (38), (39); there is gas poisoning by pulmonary irritants (40). The question

arises: "Is the polycythemia simply due to a separation of plasma from the circulating blood and its appearance as edema fluid, or is it the expression of the fact that the arterial blood is dark in color as would be the case at high altitudes?" To this question there has never been any complete answer. During the War, Goldschmidt and Wilson convinced me of the view held in the United States that, at the acute stage, concentration of the blood was due to the percolation of plasma, from the blood into the lung. The blood of convalescent patients was polycythemic for weeks and months after they were gassed. Why was this fluid not made good by the body? Probably because the arterial blood was unsaturated(41). Lastly, there are cases of short circuiting in which for one reason or another some blood which has not been oxygenated reaches the left side of the heart. The reason may be that part of the lung is not fully aerated, or it may be that the separation between the right and left sides of the heart is incomplete. This latter case is from the present standpoint most instructive. From the philogenetic point of view it represents the more primitive condition; as regards the mechanism of the body, it is less complicated by considerations which deal with the respiratory center. Such a patient does not, by breathing more frequently or more deeply increase the oxygenation of the blood, therefore there is no point in augmenting the respiratory rhythm and the fact that increased irritability of the respiratory center is mitigated by increased buffering of the blood may be of real significance. Though attention is naturally focussed on the respiratory center, one must recollect that it is but a single tissue out of a thousand whose hydrogen ion concentration needs adjustment. Wherever activity is taking place there is the tendency toward increased acid production under anoxemic conditions. The natural palliative to a too rapid acid production would be increased buffering of the tissues themselves, and the increase of hemoglobin in the blood may have a significance as a buffer because the blood buffers the tissues.

The significance of a diversity of hemoglobins. Hitherto we have treated of hemoglobin as though it were of invariable composition and always of the same properties. Yet there is abundant evidence that it is not so. We may put aside the older observation of Bohr (42) and others which were made while as yet men were ignorant of the degree to which the properties of hemoglobin depend upon those of the medium in which it is dissolved. Within the last fifteen years the following facts have come to light: Krogh (43) pointed out that the relative

affinities for oxygen and CO differed in the bloods of different animals. Douglas, Haldane and Haldane (44) have pointed out that the bloods of different individuals—both men and mice—differ in respect of the equilibrium constant of the reaction

$$Hb\,CO + O_2 \rightleftarrows HbO_2 + CO$$

and they adduce reasons for supposing that this difference is caused by a difference in the chemical properties of the hemoglobins, and not merely by a difference in the corpuscles in which they are found. Dale (45) who formerly held the opposite view, has shown that the albumins of the duck and hen are immunologically different, as indicated by their power of producing anaphylactic shock. The immunological difference is clearly to be attributed to the disparity in the globin portion of the molecule.

Landsteiner and Heidelberger (46) working at the Rockefeller Institute in New York, have shown not only that there are serological differences between the hemoglobins of different species, but that these hemoglobins are so distinct as not to influence one another's solubilities in water.

It is well known that the pigment of different animals crystallises with different degrees of facility, and Reichert and Brown (47) have made an exhaustive study of the differences in crystalline form of hemoglobin from the blood of different species.

Vlès (48) in several papers has shown that the hemoglobins of various forms of life present different constants when studied with the spectrophotometer confirming and extending the work of Sorby (49) on Planorbis.

Vlès discusses two possible explanations of the differences which he observed, *1*, that the hemoglobin may be the same in all animals, but that it may be mixed with varying quantities of hematin; *2*, that the proteins in the different hemoglobins differ and so modify the spectra.

We are face to face with the question: what is the significance—if any—of these various dissimilarities? The following observations may be of interest in this connection as relating to certain spectral properties with the affinity of hemoglobin for gas (50).

1. The position of maximum intensity of the α band of oxyhemoglobin differs in different animals. Let us call it as measured in Angstrom units A.

2. The position of maximum intensity of the CO band (B) also differs in different animals.

3. There is no evident relation between the displacements of the two bands, thus in Arenicola they are both displaced toward the blue as compared with human blood. In Chironamus, they are displaced toward the red while in the earthworm, the $\alpha - O_2Hb$ band is displaced toward the blue and the $\alpha - CO$ band toward the red. In Planorbis the $CO - Hb$ band is almost identical in position with that of man, whilst the $O_2 - Hb$ is 18 Angstrom units nearer the blue, than the corresponding band of human blood. Thus it happens that the distance between A and B, i.e., the number of Angstrom units through which the band moves when the oxygen is completely displaced by CO, is very different in different animals. This distance A to B we call for convenience the "span." Similar though perhaps not such great disparities in the "span" may be found between the bloods of different species of mammals and even between different members of the same species—notably mice.

4. As has been observed, the value of K in the equation

$$[Hb\ CO]\ [O_2] = K\ [HbO_2]\ [CO]$$

also differs.

5. If the length of the "span" in Angstrom units be plotted against the logarithm of K the points appear to be on a curve, which though in view of the large experimental error cannot positively be asserted to be a straight line, is indistinguishable from one. At all events, that is true over the range involved in the determinations which are shown in the following figure.

6. No corresponding differences are to be found in the spectra of the hemochromogens derived from the hemoglobins shown in the above diagram—a fact which supports the view of Vlès that the spectral differences are due to diversity of the protein moiety.

Granting that there are different hemoglobins in different animals, and that the difference lies in the protein, it is of second rate importance whether there are as many hemoglobins as there are species or whether there are relatively few hemoglobins mixed in varying proportions. On either assumption it would be possible to explain such a figure as the above. The important point is this—starting with a substance of definite and invariable properties such as hematin, nature can vary its power of uniting with gas—which is its significant property physiologically—by attaching to it a protein; further than this she can vary the significant property almost continuously and over a very considerable range, by modifying the protein to which it is united.

In the case of any particular hemoglobin the value of K depends upon its relative affinities for oxygen and CO; of these two the latter has no physiological significance but research on Arenicola has shown that even outside of the corpuscle the affinity of hemoglobin for oxygen in different animals differs. Here there is a new light on the chemical basis of natural selection, one which challenges research not merely in the mammalia but wherever hemoglobin is found throughout the

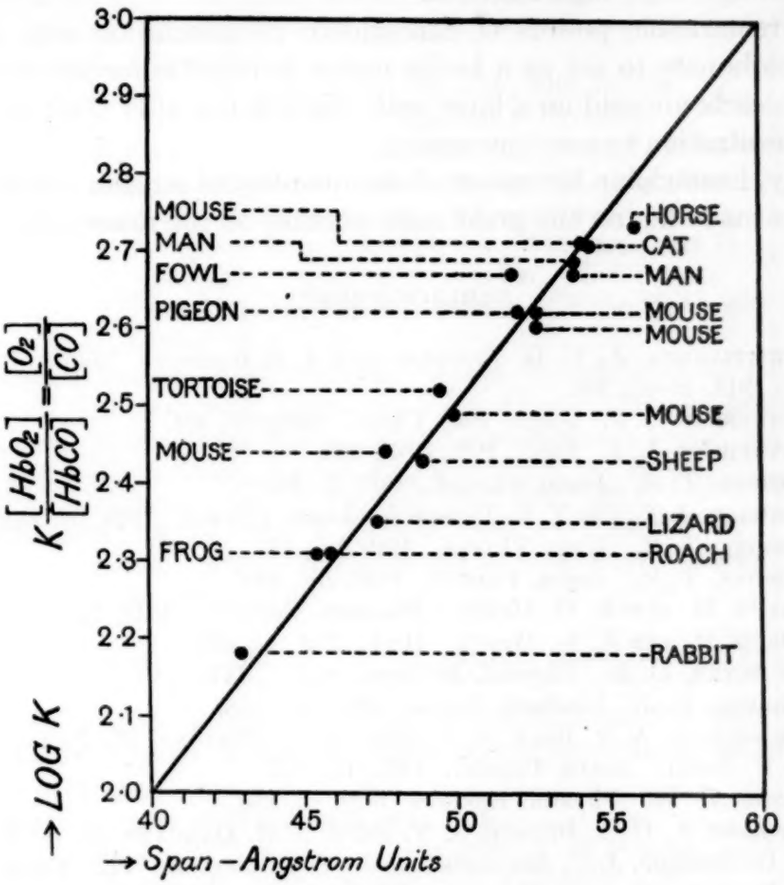

Fig. 4

animal kingdom. That challenge will be taken up in a future article. To summarize, then, hemoglobin consists essentially of an iron containing-portion which can unite with, and dissociate itself from, oxygen. By the coupling of protein to the hematin, the power of hemoglobin to acquire and part with oxygen is adjusted to the needs of living organisms so that either phase of the reaction may take place by the mere exposure of the hemoglobin to a suitable partial pressure of the gas.

The properties so achieved are exploited in the mammalia in the following way. The hemoglobin is present in large quantities in the circulating fluid, where it is enclosed in corpuscles the inside of which is maintained at a suitable hydrogen ion concentration and which are at a suitable temperature. Adjustment of the reaction within the corpuscles enables the blood to alter its power of taking up or releasing oxygen and thus to adjust the properties of the body to those of its surroundings, e.g., high altitude.

The remarkable powers of hemoglobin in association with oxygen and bicarbonate to act as a buffer makes it possible for the tissues to produce carbonic acid on a large scale and yet not alter their hydrogen ion concentration to a serious extent.

Lastly, hemoglobin by reason of the quantity of oxygen it can transport has made life on the great scale possible to the mammalia.

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INTESTINAL AUTOINTOXICATION

WALTER C. ALVAREZ

George Williams Hooper Foundation for Medical Research and the Department of Medicine, University of California Medical School, San Francisco

The idea that disease can be produced by the absorption of foul material from the intestine, especially in the presence of constipation, is one of the oldest in medicine. The layman has taken to this idea very kindly because it seems so rational. Besides, today he can hardly escape. No sooner does he begin going to school than there appears the school nurse who is likely to hold up to him the bogey of autointoxication. Later his doctor will ascribe many of his complaints, large and small, to intestinal poisoning; and every newspaper and magazine which he picks up will tell him of the terrible results that will follow if he fails to buy so and so's laxative pills, patented syringes, paraffin oil, yeast, sour milk, agar or bran.

Naturally, the literature on the subject together with its ramifications is enormous, and no complete review of it can be attempted, perhaps fortunately, since much of it is so unscientific or pseudo-scientific that no comment upon it would be fitting in a journal like this.

There is another large section of the literature which the writer will not attempt to review, i.e., the reports of experiments in which intestinal contents, fecal extracts and filtrates, urine, etc., were injected into animals. Most of this work was done very crudely years ago, and about all that can be said for it is that the intestinal contents are more toxic under certain conditions than under others. Combe (1908) and Bouchard (1894) based much of their theorizing upon the results of such experiments. See also Roger and Garnier (1905), Rodella (1914) and others.

The great interest in the use of large medicated enemas during the middle ages was due largely to the teachings of Johann Kampf, who believed that all disease was due to fecal impaction. The next wave of interest in this subject was due largely to the writings of Bouchard (1887), Combe (1908) and Albu (1895). Another wave was started by the theorizing of Metchnikoff (1905), (1908), and still another by the spectacular surgery of Sir Arbuthnot Lane (1915).

Confronted with the mass of literature which has developed around the subject, it is hard to know how or where to begin. Any city consultant will doubtless admit that the diagnosis of intestinal toxemia is being made too carelessly by many physicians, who use the term as a convenient cloak for ignorance. If they would only study their patients more carefully and if they would make more autopsies they would soon see that their cases of supposed "autointoxication" were really cases of tuberculosis, cardiovascular-renal disease, hypothyroidism, cancer, etc. To be sure, Lane will answer that tuberculosis will not spread in man if his resistance is not lowered by intestinal stasis; and actually, with such arguments, Lane and his followers link up almost every human disease with stasis. A little thought, however, will generally show how vulnerable their arguments are. For instance, as Adami has pointed out, Lane surely will not claim that the cow with her ten or twenty bowel movements a day suffers from constipation, or from enteroptosis and bands due to the assumption of a faulty posture, and yet, as we all know, she is very susceptible to widespread tuberculosis.

This article might be filled with such refutations of the arguments of the autointoxication enthusiasts but we can probably use our time and space to better advantage. The reader who would like to go more deeply into that phase of the subject should turn to articles by Clendenning (1915), Adami (1899), (1924), Hurst (1913), Woolley (1915) and Alvarez (1919).

Although we may be impressed by the weakness and futility of many of the arguments in favor of autointoxication, we must not lose sight of the fact that there are many things which make it appear very probable that poisons elaborated in the digestive tract can get through the mucosa and can injure the individual. Most of us know from personal experience that the retention of feces can make us very uncomfortable and that their expulsion can give us prompt relief. We know that we can be severely poisoned at times by toxins like that of B. botulinus, produced outside of the body by the action of bacteria on proteins. We know also that active poisons can be formed in the presence of high intestinal obstruction; and we know that the intestine is swarming with bacteria, many of which can produce serious disease. We know also that there are a number of diseases such as epilepsy, migraine and melancholia, which are often associated with constipation, and in which temporary relief can sometimes be obtained by cleaning out the bowels.

A little thought will show, however, that certain points must be proved before we can unreservedly accept the theory of intestinal autointoxica-

tion as a cause of disease. It is not sufficient simply to show that toxic substances can be formed during the enzymatic or bacterial destruction of various foods. It must be shown also that these processes take place in the digestive tract; that the substances are formed in sufficient amounts so that they can give rise to either acute or chronic poisoning; it must be shown that they are not immediately broken down further into harmless or comparatively harmless substances; it must be shown that they can pass through the mucous membrane, normal or diseased; and it must be shown that they can escape destruction in the liver. It must next be demonstrated that the repeated injection of small doses of these substances into animals or into man will produce symptoms or pathological changes similar to those supposedly produced by constipation in man. Even then doubts must arise, because the slow absorption of small amounts of material arriving by way of the portal or hepatic veins is a very different thing from the absorption of a syringeful injected once a day into an arm or ear vein.

Unfortunately, a review of the literature on the toxins which have been brought forward by various investigators as possible offenders shows that very little exact work has been done along the lines just proposed. The need for such caution in discussing the intestinal absorption of toxins can easily be seen when we remember how many substances there are which are active when injected intravenously but which are quite harmless or inert when taken by mouth.

Thus we know that diphtheria antitoxin is useless when given by mouth. Ransom (1898) found that a guinea pig can take 300,000 minimum lethal doses of tetanus toxin by mouth without showing any disturbance. Practically all of this poison can be recovered from the stools. Denys and Sluyts (1893) found that the poison produced by the cholera vibrio has no effect when put into the bowel but it has a tremendous effect on the mucous membrane of the intestine when it is injected into the blood stream. (See also Besredka, 1922.) Dixon (1902) found that strong solutions of cocaine or nicotine did not affect a frog's stomach when painted on the mucous membrane but they had powerful effects when painted on the serous side. Gayda made similar observations. Whipple (1919) found a substance in obstructed loops of small intestine which was highly toxic when injected into animals but innocuous when given by mouth or when put into loops of intestine. Hochen and Mills (1923) found that insulin has no effect on dogs or men when given by mouth or when injected into the rectum in large doses. It is promptly absorbed when injected into loops of small bowel

in the rabbit but its effect is much less marked than if it were injected into the blood stream. More recently Murlin et al (1924) have found that it is absorbed from the digestive tract in some men and women who perhaps have little trypsin in their duodenal contents. Rees and Whitehead (1923) found that the injection of pituitrin into various parts of the bowel will have no effect on blood pressure but the component which stimulates the uterus is promptly absorbed from the duodenum. Large doses of tuberculin can be given by mouth to tuberculous calves without any sign of a reaction. Enormous doses of salvarsan can be given by rectum without producing much effect. It is remarkable that even such molecules as manganese (Kobért, 1883), magnesium sulphate, and lactose are almost unable to pass through the intestinal mucosa. Mills (1923) gives the literature on the possible passage of unchanged proteins through the bowel wall and shows that it is rather conflicting. He obtained very definite evidence that as large a molecule as tissue fibrinogen can go almost immediately through the mucous membrane of the bowel. Recently Churchman (1924) has found that solutions of gentian violet, when introduced into closed loops of bowel will pass through the wall in sufficient amounts to stain gauze lying on the outside.

These observations show how carefully we must study the intestinal absorption of the various toxins which from time to time are suggested as causes of autointoxication. As we shall see later, it is probable that our greatest protection from the various toxic products of digestion resides in the intestinal mucosa.

Probable causes of poisoning. Perhaps the best thing to do first will be to review the list of those substances which have been brought forward at various times as possible sources of intestinal poisoning. Reviews of the subject with extensive bibliographies will be found in articles by Weintraud (1897), Gerhardt (1904), Ellinger (1907), Herter (1907), Wells (1920), Dixon (1913), Barger (1914), Woolley (1915), Kukula (1901) and Friedenwald (1924).

We cannot make a very satisfactory classification of these poisons because we know so little about them, but it may be helpful to think of their possible sources. Following the classification given by Weintraud and by Wells poisons may perhaps arise:

I. From constituents of the digestive secretions: pepsin, pancreatic ferments and bile.

II. From the products of normal digestion:

a. From proteins: proteoses, peptones, ammonia, hydrogen sulphide.

b. From carbohydrates: oxalic acid, fatty acids, acetone, methane.

c. From fats: fatty acids and glycerol.

III. From products of putrefaction and fermentation:
  a. From proteins:
    1. From the aromatic radicals—indol, skatol, phenol, cresol, and the pressor bases.
    2. From the fatty acid radicals—fatty acids, acetone, ammonia, cadaverin, putrescin, ethylidendiamin, isoamylamin.
    3. From the sulphur-containing radicals—hydrogen sulphide, methylmercaptan, ethylmercaptan, ethylsulphide,
  b. From carbohydrates: fatty acids, cholin, neurin and muscarin-like bodies.

IV. Synthetic products of bacterial activity in the bowel. A discussion of those toxins like that of B. botulinus which are formed outside the body would be beyond the scope of this article.

I. THE CONSTITUENTS OF THE DIGESTIVE FLUIDS. This subject needs only a brief consideration because although trypsin and bile salts are quite toxic when injected intravenously, we have no evidence that they cause any autointoxication. These substances are re-absorbed to a certain extent from the digestive canal but the body seems well able to take care of them. As we shall see later, the bile may have much to do with opening up the mucous membrane to the passage of harmful substances.

II. PRODUCTS OF NORMAL DIGESTION. *Proteins, proteoses and peptones.* The literature on the toxicity of these substances is not perfectly clear. An introduction to it can be obtained through articles by Mills (1923), Woolley (1915), Gibson (1913) and Wells (1920). Wells concludes from the work of Underhill, Gibson and Zunz that the proteoses are definitely toxic when injected into animals. Their repeated injection into rabbits may possibly produce cirrhotic changes in the liver but similar experiments with dogs and guinea pigs have given negative results. Woolley, De Mar and Clarke (1915) found that comparatively large doses of albumoses can be injected intraperitoneally in guinea pigs with practically no result.

It seems probable that the disturbances listed under the term allergy are due to the absorption of unchanged proteins, or of their toxic split products. The path may be opened up through the mucous membrane by some hereditary defect, by ulceration, by local atrophy or by the presence of the larger animal parasites. Macallum (1924) has shown recently that globules of egg protein can be followed with the microscope through the intestinal mucous membrane and into the centers of the villi.

Many physicians and laymen have the idea that there is something harmful about proteins and particularly about proteins of animal origin.

It is suggestive that monkeys and some herbivorous animals will show signs of poisoning when they are transferred to a meat diet (Herter and McCarrison, 1922); and recently it has been shown that nephritis and arteriosclerosis can be produced in rabbits by giving a high protein diet (Newburgh and Squier, 1920; Newburgh, 1919; Newburgh, 1923; Newburgh and Clarkson, 1922). It was shown years ago by Garnier and Simon (1907), (1908) that rabbits die in a few days when put on a raw meat diet. To a large extent, they probably die of starvation as metabolic studies indicate that they cannot utilize the end products of the protein digestion. They also develop very promptly a septicemia (Garnier and Simon, 1907). Man himself apparently cannot exist very long on a diet of lean meat alone. Stefansson (*My life with the Eskimo*, p. 140) found that he had to have some fat with it in order to maintain his strength.

The best proof that a protein diet is not necessarily harmful has now been given by Osborne and Mendel (1924) who have reared large numbers of rats on diets containing no carbohydrates at all and protein up to 85 per cent. The animals grew very well and the only change found at autopsy was what seemed to be a purely functional enlargement of the kidneys.

One objection to many of the arguments brought forward against the use of meat is that man seems originally to have been a carnivorous animal because he was a hunter, a fisherman and a herdsman for thousands of years before he was much of a husbandman. Rink has pointed out that during the seal-catching season the Eskimos eat 4 kilos or more of meat a day; and until the diseases of civilization were taken to them, they were a very healthy people. That white men can live in the same way was shown by a party of six antarctic explorers who lived comfortably for six months on seals and penguins, and by Stefansson who lived on meat and fat for many months at a time.

In recent years the work of Folin and his associates (1912), (1922), has shown that the *amino acids* produced during the digestion of proteins are absorbed unchanged through the intestinal mucosa. They go through the liver and apparently are used in the tissues. It would seem, therefore, that we cannot ascribe intestinal autointoxication to the absorption of amino acids. The well-known fact that Eck fistula dogs cannot stand much meat shows that the liver must serve to protect the body from the harmful action of some substances coming from the bowel (see Hahn, Massen, Nencki and Pavloff, 1893). As Folin says, the Eck fistula dog seems to furnish the first really definite illustration

of "autointoxication by way of the large intestine." He feels, however, that as yet we do not know what the poisons actually are.

Ammonia. Folin (1912) has shown that ordinarily there is a little more ammonia in the portal vein than in the general circulation. He has shown also that it comes almost entirely from the large bowel where it is probably formed during the putrefaction of protein. The amounts of ammonia in the portal blood are so small that Folin thinks it is extremely unlikely that it could produce any disturbance even in an Eck fistula dog. Rovighi (1898) and Joannovics (1904) injected ammonium carbonate and ammonium carbamate into animals and obtained degenerative changes in the liver, but their experiments were done with large and rapidly toxic doses, and we cannot apply their results directly to man. Herter (book, p. 221) came to the conclusion that ammonia is probably never formed in sufficient quantities in the bowel to render it a factor in autointoxication. He felt that our protective mechanisms against this substance are so good that we are not likely to suffer from any excess production of it.

Hydrogen sulphide. The literature on this subject is not entirely in agreement and it might be well if some more work were done upon it. Brunton and Cash (1886) found that it is readily absorbed by the blood but it is so rapidly excreted by the lungs that they thought it could hardly reach the nerve centers in sufficient concentration to produce toxic symptoms. Claude Bernard found it very toxic when absorbed by the lungs but quite harmless when given intravenously, subcutaneously, or by the intestines. Herter (book, p. 227) thought that this substance might be of importance in autointoxication but he admitted that we have no knowledge of the effects upon the organism of the small amounts which are constantly found in the colon. He found that this gas will poison dogs when it is injected into their colons. If large amounts are given, fatal collapse may occur. Herter (1907) and Flint point out, however, that many persons subjected to the Burgeon treatment for pulmonary tuberculosis have taken considerable quantities of hydrogen sulphide by rectum without any signs of injury. Wells (book, p. 590) gives the normal amount in 100 grams of feces as between 58 and 66 mgm.

From carbohydrates: According to Herter (book, p. 214) the products of fermentative decomposition of carbohydrates may be dismissed with a few words. The volatile fatty acids are irritants and they might produce some disturbance in a person with an irritable stomach or small intestine. They apparently do cause quite serious upsets with diarrhea in the much more sensitive infants and children.

There is still another way in which these acids might cause trouble and that is by robbing the organism of some of its bases. Thus, Dixon (1913) points out that an excessive formation of lactic acid in the bowel may increase the normal excretion of calcium four or five times. Such calcium starvation might seriously inconvenience a growing body. Porter, Morris and Meyer (1919) have made similar observations

Acetone is a substance which might possibly cause trouble but Herter (book, p. 219) doubts whether it has any significance.

Oxalic acid. This is perhaps the most important toxic substance which can be formed from carbohydrates. The older literature on the subject can be found in a splendid review by Kobert (1906). When Donne, in 1838, discovered calcium oxalate crystals in the urine many thought that the so-called oxalic acid diathesis might explain a number of syndromes. Later, however, oxalates were found in every urine examined. According to Baldwin (1900) the average amount excreted in twenty-four hours by healthy men and women is about 10 mgm. This generally disappears as soon as the individual is placed on a diet free from oxalates and oxalic acid, showing that these substances are formed from the food and not during metabolism. On a diet containing a good deal of rhubarb, Baldwin found as much as 67 mgm. of oxalic acid in the urine. She feels that even these large amounts can hardly have any significance because in the last century oxalic acid was used extensively in making lemonade and was considered to be harmless. According to her, Piotrowski, on several occasions, took from 4 to 7 grams in twenty-four hours without experiencing any discomfort, and once he took 8 grams within an hour. (See also Rosenau, 1919.) Wells agrees with Miss Baldwin that the small amounts of oxalic acid formed in the body are probably harmless.

Lieb (1923) has recently attempted to revive the subject but he brings forward no new data and his argument is based almost entirely on the results of treatment intended to diminish the amount of oxalic acid formed in the colon.

Products of putrefaction of protein. As Herter says (book, p. 220):

> When we turn to the consideration of the nitrogen containing and sulphur containing products of putrefactive cleavage, the scantiness of our knowledge comes into view with almost discouraging clearness. That putrefactive processes are attended by the formation of bases such as ammonia, amins, diamins, ... cholin, neurin, sulphur compounds and various aromatic bodies has been known many years. . . . . When, however, we ask ourselves what we can safely say of the conditions under which such substances arise in the human intestine and of their pathological effects, we are able to give in most instances only very inadequate answers.

Indol. This is one of the most carefully studied of all the substances which have been brought forward as possible causes of autointoxication. Much of the work has been done with its derivative, indican, which was well studied by Jaffe in 1877. He found in dogs that its excretion is dependent on the nitrogen intake. He observed the now well-known fact that it appears in larger amounts with obstructions of the small intestine than with obstructions of the large intestine. He found also that the retention of feces for days or weeks does not necessarily increase the amount of indican in the urine.

Unfortunately, much of the enormous literature on indicanuria is of little value either because no quantitative methods were used or because they were inaccurate. Nesbitt (1899) found that on the average we excrete about 12 mgm. of indican a day. Borden places the figures around 6 mgm. Amounts up to 20 mgm. were found in men and women who seemed to be perfectly well.

The next question is: What is the toxicity of indol? The first to attack this problem was Nencki in 1876. According to Herter, he found that a dog showed no signs of poisoning when given 1 gram of this substance by mouth but developed diarrhea and hematuria when given 2 grams in the twenty-four hours. Rovighi (quoted by Herter, 1898) found with rabbits that indol and skatol produced torpor, widespread paresis, feeble heart action and lowered temperature. The fatal dosage of these two substances for rabbits was found to be from 1.5 to 2 grams, given subcutaneously in the course of forty-eight hours. This work was repeated by Herter (1898) who found that intravenous injections of indol are markedly toxic to the nervous system, both in rabbits and dogs. The repeated daily injection of small quantities of indol into rabbits caused profound disturbance of nutrition, ending in death in the course of a few weeks. The total quantities used varied between 130 and 220 mgm.

Woolley and Newburgh (1911) performed similar experiments with rabbits and white rats, and produced hypertrophy of the adrenals and slight interstitial changes in the kidneys. It must be remembered that the dosage used in most of these experiments was much greater than that ordinarily found in the intestine of man. Nesbitt (1899), who injected indol in 0.1 gram doses into the veins of dogs, found no changes in arterial pressure and came to the conclusion that "twenty times as much of either indol or skatol as are secreted daily by a man of 70 kilos weight may be injected at one time into the jugular vein of a dog of four kilos without producing an appreciable effect on either circulation or respiration."

Herter (1898) gave 5 cc. of a 0.1 per cent solution of indol to a small ring-tailed monkey daily for two months without obtaining any definite result.

As it is very unsafe to apply the results obtained with large doses in rabbits and other animals directly to the problems of man, Herter gave indol by mouth to a number of healthy men. He found that they were able to take without discomfort amounts much greater than those ever found in the bowel. Thus, one man took 1 gram in divided doses during the day without showing any symptoms. Two grams produced restlessness, an overly active brain, insomnia and headache. Other subjects had slight frontal headache and slight dizziness. Sometimes there was a sense of fatigue. One man experienced no discomfort until he had taken large doses for several days. The dosages used were much greater than those that have ever been found in the bowel.

Herter states in his book (p. 242) that he has probably under-estimated the importance of indol as a toxic agent in man. He says that as much as 50 or 60 mgm. can sometimes be found in 100 grams of fresh feces, and he thinks such amounts might produce symptoms. He thinks this is the only known aromatic product which is absorbed in sufficient quantities to have a toxic effect on the neuro-muscular system. It should be noted, however, that Herter (book, p. 92) found that the epithelium of the digestive tract is able to bind indol in large amounts so that the substance cannot be recovered by distillation. This is probably one of the main protective mechanisms of the body against its action. Adami (1914) thinks that indol and skatol are probably not so easily absorbed from the large intestine as we have hitherto thought because it is possible to obtain large amounts of indol from the stools when the urine is almost free from indican. Wells also feels that the amounts of indican found in the urine ordinarily are so small in proportion to the doses used for experimental injections, that it is very doubtful whether this substance has any practical importance.

There is considerable evidence, however, that amounts of indol which might be harmless to one animal or man might be harmful to another, on account of the lowered ability of the tissues to oxidize the substance. This was shown by Richards and Howland. They found that substances like anesthetics and KCN, which interfere with tissue oxidations, greatly increase the susceptibility of animals to the harmful action of indol. Herter thinks therefore that indol may perhaps do more harm in the aged than in the young.

There is considerable evidence also to show that a large amount of indican in the urine may mean simply that we are dealing with a severe renal insufficiency. That indican is not produced solely in the bowel is emphasized by Mellanby who says that when he wants to demonstrate indican to his students, he tries to get a sample of urine, not from a constipated individual, but from a man with carcinoma of the liver.

The efforts to show that indicanuria is always marked in certain diseases have failed repeatedly. As Hale White says, there is no doubt that an excess of indican is often associated with serious intestinal disturbances and with conditions suggesting auto-intoxication, but we know also that many people pass large amounts of indican for years and yet remain in good health. Furthermore, large numbers of constipated people show no increase in the indican in their urine.

We see, then, from all this, that although indol has toxic properties and although it can produce in man some symptoms similar to those observed in the constipated, it must not be forgotten that some of those symptoms do not correspond to those observed clinically; and what is more important, the experiments have been done with doses enormously greater than those met with even in the presence of disease.

Skatol (Herter, book, p. 239). As compared with the quantity of indol in the intestine, the quantity of skatol is almost always small. Herter has occasionally seen amounts as high as 8 or 10 mgm. in 100 grams of fresh material. He observed this only in persons with marked signs of intestinal or nervous disorder. In one of these cases the patient's condition suggested chronic alcoholism. Skatol behaves in the organism much like indol as respects its toxic properties but it is somewhat less poisonous.

According to Brieger, skatol is absorbed from the bowel only in traces. According to Harley (1913) it is acted on in the liver and eliminated as an aromatic compound. One difficulty in the way of the absorption of skatol may be its slight solubility in water. Porcher and Hervieux (1905) rubbed up skatol in oil and gave it to animals by stomach tube. They found it present normally in the urines of a number of animals and they feel that it is not possible to connect it up with any particular disease. Okhoubo, working with Metchnikoff (1910), injected skatol repeatedly into rabbits and could not produce any arteriosclerosis even in these extremely susceptible animals. He did get arterial changes with indol.

Indol-acetic acid appears in the normal urine in minute traces. Ross (1913) found it in the urines of 21 per cent of normal persons but in 48 per cent of some dementia praecox cases.

PHENOLS AND CRESOLS. Some of the older literature in regard to phenol will be found in an article by Nesbitt. He doubts whether it is of significance as regards autointoxication because it has been used for a long time as a medicine and has been given in amounts much greater than those found in the bowel. Küster (1879) and Falkson (1881) give the results of analyses of the black urines seen so commonly in the early eighties when patients and surgeons were heavily exposed to the action of carbolic acid sprays and compresses. Falkson found 2 grams of phenol in his urine during the twenty-four hours following an operation. Next day there was 1.3 gram and the day after that there was 0.65 gram. On the first day he was tired and had a little headache but after that he felt well. As we shall see later, the usual amount in the urine varies between 0.2 and 0.4 gram a day.

Dixon (1913) feels that the phenols are so little poisonous and the body is so well able to protect itself against them that they are probably not very significant. Herter (book, p. 238) thought it likely that the continued absorption of moderate quantities of phenol from the bowel over a long period of time might damage the liver.

The best recent work on the phenols has been done by Folin and Denis (1915) and Dubin (1916), (1917). They also review the early literature. They found from 200 to 400 mgm. of phenols in the urine per day. Much more is undoubtedly formed in the bowel, but from 7 to 20 per cent of that amount is eliminated with the feces. The most important phenol in the urine, at least quantitatively, is paracresol. The amount of phenol in the urine does not necessarily parallel the amount of indican because the two come from different sources.

The striking contribution to the subject made by Folin is that conjugation is not so complete as previous writers have thought it to be. The free phenol represents ordinarily from 30 to 90 per cent of the total excreted, but in some children he found no conjugation at all. This observation was confirmed by Dubin (1916). He found that with an Eck fistula the free phenols in the urine run as high as 97 per cent. The making of intestinal obstructions increased the amounts of both free and combined phenol. He thinks that the amount of phenol in the urine can be taken as an index of intestinal putrefaction.

Nesbitt found that the cresols are less toxic than the phenols and recovery from poisoning by them is more prompt. Thus the injection of 50 cc. of 0.5 per cent cresol into the veins of a 4 kilo dog caused the blood pressure to fall 41 mm. but it was back to normal in thirteen minutes. According to Denny and Frothingham (1914), the long-continued feed-

ing of paracresol to rabbits produces no definite effect. See, however, Metchnikoff (1910) who apparently produced arteriosclerosis in this way. Theis and Benedict (1918) measured the phenols in the bloods of 83 hospital patients and found amounts between 2 and 8 mgm. per 100 cc. The average was 4.7 mgm. There was no characteristic increase in any disease with the possible exception of hernia and sarcoma.

Histamin. In 1910 Ackermann showed that histamin could be produced from histidin through the action of bacteria. This was confirmed later by Berthelot and Bertrand (1912), Mellanby and Twort (1912), Mutch (1914), (1915), and Koessler and Hanke (1924). The earlier writers thought they were dealing with a special bacillus which was present only in diseased persons, but it has since been shown that there are a number of bacteria present in the normal bowel which can produce histamin under certain definite conditions. According to some, the medium must be acid; it must contain oxygen, and there must be some glycerol or glucose present. Others think that histamin is never produced if carbohydrates are present. When one reads how carefully conditions have to be prepared in order to get a good yield of histamin one wonders whether they would ever be maintained that way in the bowel for any great length of time. Mellanby (1916) found also that he had to use pure cultures of certain bacteria in order to get histamin because if other bacteria were present they broke the substance down as fast as it was formed. His studies indicated that it was not absorbed to any appreciable extent form the colon but was destroyed there by relays of bacteria.

Similar conclusions were reached by Meakins and Harington (1921), who found minute amounts of a substance resembling histamin in the cecal contents of six men and women with artificial ani. No histamin could be found in their feces showing that it was destroyed on its way through the colon.

Mutch (1914), (1915), who did considerable work in support of the views of Lane, found a histamin-producing bacillus in some of the pieces of bowel removed by Lane at operations. From observations made on fourteen cases he felt that he had "proved definitely" that this substance is responsible for the low pressures seen in some cases of autointoxication.

Since then Abel and Kubota (1919) have found a substance giving many of the reactions of histamin in some of the normal tissues of the body and in many foods. Abel's most recent work (1924), together with that of Clark (1924), shows that this is probably some precursor (not histidin) from which histamin can very easily be formed. Abel

has suggested that this substance may have a considerable physiological importance: acting as a tonic to the intestinal muscle and a dilator of the capillaries in those places where more blood is needed for digestion.

It seems quite probable that some histamin is formed in the bowel. The next question is: can it get through the mucous membrane and can it get through the liver in amounts sufficient to affect the body? Meakins and Harington (1922) injected a 1 per cent solution into various parts of the bowel of cats and found that it was absorbed quite well from the ileum and to a slighter extent from the cecum. The effects were less striking when food remnants were present to dilute or adsorb the injected material.

Popielski (1920) got no effect from injecting histamin into the duodenum but the effect of injection into the mesenteric veins was about the same as that obtained by injection into the crural veins. He felt therefore that the main barrier to the action of histamin is in the mucous membrane of the bowel. Koessler and Hanke (1924) have come to the same conclusion. They feel that most of it is destroyed in the intestinal mucosa and only a small part is taken care of by the liver.

Oehme (1913) found that an animal can stand two or two and a half times the dose injected into the portal vein than it can stand injected into the ear vein. He found also that an animal can stand large doses without showing any symptoms if they are injected slowly over a period of an hour or so. There was evidence also that with repeated doses the body becomes more and more efficient in getting rid of the poison. Fühner (1912) confirmed this ability of the body to become accustomed to the drug. This point must be faced by those who would ascribe low blood pressure and other symptoms in man to chronic poisoning by small doses of histamin. According to these results of Oehme and Fühner, small doses slowly absorbed would have no effect on the blood pressure, or if they did at first, such effects would promptly disappear. The inference therefore must be that if histamin, absorbed over long periods of time, is ever toxic to man, it probably shows itself in some way different from those seen in the acute poisoning experiments.

Another difficulty in the way of applying the results of pharmacologists' research on animals directly to the problems of human disease is brought out by McDowall (1923) and Geiling and Kolls (1924) who found a considerable difference in the action of histamin when they varied the size of the dose and the depth of anesthesia used. Any pharmacologist knows that we cannot always assume that the results of an almost lethal dose of a drug given to an anesthetized animal are going to be the same

as those of repeated small doses absorbed through the portal circulation of a man. The results might be just reversed. Unfortunately this point is missed by most clinical writers on the subject.

Meakins and Harington (1922) did a number of perfusion experiments which confirmed the results of Dale and Laidlaw (1911) as to the slight destruction of histamin in the liver. To be sure, such negative experiments do not prove that the normal liver in situ cannot handle the problem. With Eck fistula animals they obtained some results suggesting strongly that the liver does play a part in diminishing the toxic effects of the drug. Damaging the mucous membrane of the bowel lessened the absorption of the drug.

Schenck (1921) gives the literature on the effects of histamin on man. On injecting subcutaneously 2 mgm. he produced very severe symptoms, such as a rushing of blood to the head, nausea, and cramps of the bronchial musculature. Doses of from 4 to 5 mgm. produced a tremendous drop in blood pressure.

It is unfortunate that those who like to ascribe a number of disease states to the presence of histamin in the bowel do not read the conclusions of those men who have worked most with the substance. As we have already seen, Abel thinks that it is a more or less normal hormone of distinct value to the individual. Meakins and Harington admit that it might act as a poison, lowering blood pressure, "But, taking into account the very slow rate of absorption from the cecum and the minute concentrations in which we found it to be present, it is scarcely conceivable that any ill effects could be produced by absorption from this part of the intestine. If, however, an appreciable concentration of histamin is formed for any reason in the ileum, we should expect to observe toxic effects." "We consider therefore that the balance of the evidence so far obtained is against the view that histamin is an active agent in causing intestinal intoxication" except possibly in cases of ileocecal regurgitation.

Mellanby and others have pointed out that this drug is such a powerful stimulant to smooth muscle that if any considerable amounts were to be formed they would immediately produce diarrhea and vomiting. He thought it might possibly have some significance in the diarrheas of children but certainly not in the constipation of adults. "In fact, it seems impossible with our present knowledge of the bacterial production, bacterial destruction, and the absorption of histamin in the intestine to be able to claim any causal relationship between chronic alimentary toxemias and this substance."

Another difficulty with all this work is that low blood pressures are not found so very often in the constipated and in those who feel that they are suffering from intestinal toxemia. It would seem also that if enough histamin were being absorbed to maintain the drop in blood pressure, it would have to produce also severe attacks of asthma, diarrhea, and skin flushing.

The pressor bases. It is rather fortunate for those who like to ascribe pretty nearly everything to intestinal autointoxication that they can claim as their own not only the patients with low blood pressures but also those with high pressures. The high pressures are supposed to be due to some of the amins which are related structurally and physiologically to epinephrin. The most powerful of these substances is parahydroxyphenylethylamin, or tyramin. Less active are phenylethylamin and isoamylamin. An extensive literature on the physiological actions of these substances will be found in the monographs by Barger and Dale (1910) and Barger (1914), and in articles by Guggenheim (1912),(1913) and Guggenheim and Loeffler (1915). Another good review is by Granger (1912).

The following list taken from Guggenheim and Loeffler may be helpful at this point as it shows so graphically the relative physiological activities of these substances when tested on a strip of guinea-pig uterus suspended in 100 cc. of Ringer's solution.

	gram
Histamin	0.000,025
P-hydroxyphenylethylamin (tyramin)	0.001
Phenylethylamin chlorhydrate	0.001
Isoamylamin	0.005
Indolethylamin	0.002
Suprarenin hydrochloride	0.000,01
Methyl guanidin nitrate	0.01
Cholin bromhydrate	0.01
Acetylcholin bromhydrate	0.000,001
Neurin bromhydrate	0.001
Fatty acid salts and oxalates	0.01 to 0.10
Indol, phenol and cresol	0.05 to 0.01
No effect was obtained with the various amino acids, methyl and ethylamin, trimethylamin, cadaverin, putrescin and guanidin in doses of	0.10

Tyramin. This substance is found in cheese and in other foods, and it seems to be the chief pressor constituent of ergot. It acts very much like epinephrin but it is only one-twentieth as active. Hewlett (1917)

injected subcutaneously from 60 to 80 mgm. into normal men and found a marked increase in systolic pressure, and an increase in the output of the heart. The effect was a little more lasting than that obtained with adrenalin. Findlay (1911) used from 20 to 80 mgm. intramuscularly. The increase in blood pressure reached its maximum within ten minutes and generally was gone within fifteen minutes. The larger doses were followed by terrific headaches. He could not get any definite results with the oral administration of the drug. Clark (1910) got similar results with the injection of from 20 to 50 mgm. into normal men. When he gave 200 mgm. by mouth he observed slight rises in pressure about an hour later. His results throw doubt on the one experiment of Dale and Dixon (1909), one of whom took 10 mgm. by mouth and obtained a rise in blood pressure within a few minutes. It may have been one of those temporary fluctuations in pressure which have been described by several writers.

Dale and Dixon (1909) produced a slow, steady rise of blood pressure by the injection into the rectum of a decerebrated cat of 10 cc. of a N/10 solution of isoamylamin hydrochloride. This base is somewhat similar in its action to tyramin but its effects are much weaker and more evanescent.

The next question that arises is: Are we justified in ascribing the hypertension which is seen in man to the intestinal absorption of these bases? There are several objections to this view which the writer feels are quite insuperable at this time. In the first place, it should be noted that the effects obtained when the drugs are given by mouth are much weaker than those obtained after intravenous injection, and very large doses have to be given. Ewins and Laidlaw (1910) and Guggenheim and Löffler (1915) found that these bases are, to a large extent, detoxified in the liver by a change into the corresponding acetic acids. It would seem that this mechanism, although not sufficient to cope with large doses injected into the bowel, should easily handle small doses trickling in gradually.

Another objection is that with repeated injections of these substances into animals, the effect soon falls off, whereas in man we know that the high level of pressure is usually maintained with remarkable constancy for many years. Furthermore, if these high pressures were dependent on the constant absorption of a drug from the bowel, we would expect to see marked drops after purgation and we know that these only occasionally take place.

Another difficulty is that, in small doses, these substances sometimes fail to produce a rise in blood pressure because they inhibit the heart through the vagi. In order to get the full pressor effect in animals these nerves have often to be cut.

Another great objection to the autointoxication theory of hypertension is the finding by Alvarez (1923) that pressures over 140 mm. of mercury are commonly met with in young men between the ages of sixteen and twenty-four. Such high pressures were found in 21 per cent of 5,300 freshmen entering the University of California. If intestinal stasis were producing the hypertension, we would expect to find a higher incidence among the women students of the same age who generally suffer more than do the men from constipation. Actually, only 3 per cent of 8,934 women had pressures over 140. We see, therefore, that some as yet unknown sexual factor must enter very largely into the problem.

Bain (1910), (1911) has studied the pressor substances which can be extracted from the urine and thinks that they are probably mixtures of isoamylamin and tyramin. He has looked for these bases in over fifty urines and has found the largest amounts in men and women with normal pressures. They were absent from the urines in almost all of the children and almost all of the men and women with hypertension. Bain believes that their absence in children is due to the fact that they are not produced, but that their absence in people with hypertension is due to their absorption. His data are too few and his work is not convincing. Furthermore, Granger (1912) has been unable to confirm his findings in 37 cases examined. Barger and Walpole (1909) once evapporated 30 liters of urine and were unable to get enough of these bases for analysis.

Harvey has given tyramin to rabbits by mouth and has injected it into them every day or every several days for periods of from a month to a year. He has found sclerotic changes in the kidneys and in some of the arteries. Similar work was done by Etienne with the pressor bases obtained from the urine. One objection to such work is that it has been done on rabbits, which are subject to spontaneous arteritis. Metchnikoff (1910) states that one of his students was able to produce atheroma of the aorta in rabbits by injecting them with phenylethylamin and isoamylamin but not by giving these drugs by mouth.

We may sum up this section by saying that it does not seem at all probable that hypertension, as we see it, lasting through a lifetime, is due to the absorption of the pressor bases. It may easily be, however,

that the absorption of these substances in amounts larger than normal and over long periods of time might produce some symptoms or some degenerative changes. What those may be we do not know. The problem is very much the same as that with adrenalin. We know from the work of Janeway and Clark (1912) and others that there is not enough adrenalin normally in the blood to account for the maintenance of blood pressures, and that there is no increase in cases of hypertension. There is considerable evidence, however, that the continued injection of adrenalin into an animal will produce degenerative changes in the aorta.

Cholin is formed in the bowel from lecithin and might possibly at times cause trouble. Abel (1919) says its action is very feeble as compared with that of histamin. Some investigators think it might be of value to the organism as a stimulator of intestinal peristalsis. Hunt (1915), Shanks (1923) and others have shown that it disappears almost immediately when injected into the blood stream. It is not excreted by the kidneys and it does not disappear if the abdominal organs are excluded from the circulation. Hunt, who has done a great deal of work with this substance, feels that we have as yet no grounds for saying that it has any pathological significance.

The danger with cholin is that some highly toxic substances may be formed from it. One of these, neurin, was investigated by Nesbitt (1899). He points out that if any large amount of it were produced in the bowel, it is such a powerful stimulant that it would probably cause diarrhea.

Magnus and his students have been working for some time on the theory that cholin is the normal hormone which helps to keep up the intestinal movements. See Arai (1922) and v. Kühlewein (1921).

Methyl guanidin. This is a highly toxic substance which has been found by Koch (1912), (1913) in the urines of dogs with tetany following parathyroidectomy. Heyde (1911) has found it in the urines of persons badly burned and he thinks it accounts for their toxemia. There seems little reason at present for believing that it is ever formed in the bowel so it will not be discussed further.

SYNTHETIC PRODUCTS OF BACTERIAL ACTIVITY. Many writers on the subject of autointoxication have been impressed by the large number of bacteria normally present in the bowel. MacNeal, Latzer and Kerr (1909) who have probably made the most careful estimate of the number of fecal bacteria excreted daily by normal adults, found that the number is about 33×10^{12}. These bacteria supply 46 per cent of the total fecal nitrogen. As they are not taken in with the food they

must grow in the bowel. Most of this growth takes place in the cecum and ascending colon where the feces are liquid enough to furnish a proper medium. There are a good many bacteria also in the terminal ileum.

With the drying out of the feces in the distal two-thirds of the colon most of the organisms seem to die out, and only a small proportion of those which appear in a microscopic field can be grown on culture media. There is evidence to show that there are many substances formed in the bowel by the mucous membrane and by the bacteria themselves, which restrain or modify their growth, so we must always be very careful how we apply the results of *in vitro* experiments to conditions in the living animal.

Excellent reviews of the subject of intestinal bacteriology can be found in the book by Kendall; in many articles by him (1910), (1911); in Schmidt and Strassburger's book, in Cheplin and Rettger's book (1920); and in an article by Morris, Porter and Meyer, (1919) A complete bibliography is given by Kendall (1911).

As has been shown by all of these investigators, the intestinal flora is quite constant in its makeup, and there is much evidence to show that it is serviceable to the host in that it tends to keep out the more harmful bacteria which might otherwise gain a foothold. Even in the diarrheas and intestinal upsets of adults, careful culturing rarely shows any change in the flora. Changes are often found, however, in the digestive disorders of infants and children, as has been shown by Escherich (1886), Kendall, Morris, Porter and Meyer, Rettger, and many others.

It should be noted also that, especially during epidemics of typhoid fever or dysentery, pathogenic bacteria can be found in the stools of many people who show no sign of disease, Thus Herter (book, p. 103) found B. pyocyaneus, B. putrificus and large numbers of B. welchii in the intestines of people who seemed to be normal; and Kendall remarks that some individuals with diarrhea and with an over-growth of harmful bacteria in the bowel can live for years without any signs of autointoxication. Another important point in regard to the intestinal bacteria is that there are very few which produce soluble toxins. Endotoxins may possibly be liberated from others but unfortunately, as Zinsser (1920) has recently pointed out, our knowledge about their constitution and the ways in which they injure the body is almost non-existent.

It has been known for many years that the intestinal bacteria can be very useful to the body, not only as a protection against invasion by harmful organisms, but by helping in the processes of digestion. For this reason there has been much discussion as to whether or not animals

can live without these bacteria. Probably the most convincing of the older observations on the subject are those of Levin (1899), who found the intestines of a number of Arctic animals sterile. More recently Loeb and Northrup (1916), (1917), have been able to raise large numbers of banana flies under sterile conditions. Their experiments show that the duration of life in these flies varies inversely as the temperature of their environment. This work offers no support to the assumptions of Metchnikoff that life is brought to an end by the toxins produced by bacterial action in the colon.

As is well known, Metchnikoff popularized the idea of stopping autointoxication and prolonging life by sterilizing the digestive tract or by changing the flora from a putrefactive to a less harmful, fermentative type. It is evident that if good results could be gotten in this way, we would have considerable proof for the validity of the autointoxication theory.

In the first place, the food might be sterilized, but it has been shown that this has little effect in reducing the number of intestinal bacteria. Complete starvation may even result in an increase of their number. Many intestinal antiseptics have been tried out and some are still being used, but practically all of those workers who have taken the trouble to make bacterial counts before and after the administration of these drugs admit that they have little or no effect. Purgation may sweep out a good many bacteria, but the resulting increase in the fluidity of the colonic contents often results in a subsequent increase in the count.

The literature is full of articles on the excellent results which can be obtained in many diseases by the use of colonic flushing, but in most cases the writers are clearly enthusiasts with one idea: men who have so little knowledge of the nature of scientific investigation and proof that their statements cannot be accepted at face value. They undoubtedly must often get good results, but they are not able to tell us when and in what type of disease.

Much work has been done in the last thirty years in an effort to change the bacterial flora by the giving of sour milk. Herter thinks that Poehl was the first, in 1887, to suggest the use of this method. His work was repeated by several men and was then taken up and popularized by Metchnikoff. Any scientifically trained man who will read Metchnikoff's book on *The Prolongation of Life* will probably stop to wonder how a man in his position could have written so positively on the basis of so little exact proof. Ledingham (1913), in his review of the subject, points out how unsatisfactory and how unconvincing also are the more

technical papers which were put out by some of Metchnikoff's pupils in an attempt to support his theory.

There is an enormous literature on the results that have been observed in various diseases after giving milk soured with the Bulgarian bacillus, but many of the arguments of these writers have been invalidated by the recent work which has shown that that bacillus not only does not replace other forms in the bowel but it does not seem even to be able to hold its own there. See Hull and Rettger (1917), Herter and Kendall (1908), and Einhorn, Wood and Züblin (1910). Apparently the earlier writers with their imperfect cultural methods fed one bacterium by mouth and recovered another closely related saprophyte from the stools (Rahe, 1915). When this type of sour milk helps, as it sometimes does, its beneficial action may be due to its laxative properties or to the lactose which tends to restrain the growth of the proteolytic bacteria in the bowel.

The use of the bacillus acidophilus, which is a normal inhabitant of the digestive tract, seems to be much more promising. Its administration was first suggested by Rotch and Kendall in 1911, and it has since been studied carefully by Cheplin and Rettger (1920), by Kopeloff (1922), (1923) and others. Good results are claimed in the treatment both of constipation and diarrhea, but sufficient time has not yet passed for us to evaluate this work.

The intestinal flora can be changed not only by giving large numbers of living acidophilus bacilli but also by giving large amounts of lactose. The lactose is so poorly absorbed from the bowel that it gets down into the lower ileum and colon and there modifies the culture medium so that the saccharolytic or fermentative bacteria can outgrow the putrefactive ones. The advantage of this is that the products of carbohydrate fermentation have fewer potentialities for evil than have the end products of protein decomposition. Another hopeful thing about the B. acidophilus is that it tends to grow more in the colon while the B. bulgaricus tends to stay in the ileum.

One has only to read the article of Porter, Morris and Meyer (1919) or the many articles by Kendall to realize the great possibilities of this type of treatment in some of the severe intoxications of children and infants. Porter describes these alimentary intoxications as follows: There is, first, a mild type, with constipation, failure to gain in weight, pallor, head sweating, and loss of turgor. Second: a fulminating, acute type with profound toxemia, shock, rapid loss of weight, and often fatal issue with acidosis; and third: a serious, chronic type, occurring usually

between the tenth and the twentieth months, characterized by frequent stools, anorexia, loss of weight, and increasing intoxication.

In the severe type of case, in which the symptoms often closely resemble those of acute intussusception, the stools always show putrefactive organisms in great abundance. Necropsy shows acute degenerative and fatty changes in the liver and kidneys. Recently Porter has been able to save a number of these children by giving large amounts of lactose solution with 2 per cent sodium bicarbonate. The older children have dark-circled, sunken eyes; they are often irritable and naughty, but at other times they may be dull and depressed. In these cases a remarkable improvement can sometimes be produced by changing for a while to a purely carbohydrate diet.

These studies show clearly that children can be chronically or acutely poisoned when abnormal types of bacteria develop in their intestines. One difficulty, of course, is that we cannot be sure, especially in the acute types, that these bacteria have not passed through the barrier of the intestinal mucosa. This possibility will be discussed later under the heading of "latent infection."

Unfortunately, we cannot say that certain processes take place in adults simply because they take place in infants and children. This is one of the great mistakes which one finds all through Combe's work. There we find a children's specialist who has become so used to ascribing the many symptoms of his little patients to the bacterial decomposition of food in their intestines that he cannot help looking at adults in the same way. We must realize that the digestive tract of the infant, changing from his mother's milk to a more or less artificial food, is much more sensitive and much more vulnerable than that of the adult. The mucous membrane is much more permeable both to toxins and to bacteria. Combe, therefore, has no right to talk about babies in one breath and adults in the next as if there were no difference between them.

Syndrome of constipation: The commonest syndrome is the well-known one which goes with constipation and which is promptly relieved by the evacuation of the bowel. It consists mainly of mental haziness, "dopiness," malaise, headache, coated tongue, poor appetite and so-called "biliousness." There is nothing imaginary about these symptoms; most of us have suffered from them at some time or another, but we can now say quite definitely that, when associated closely with constipation, they are rarely due to the absorption of any poison. The reason for this belief is that when we question these people we find that practically all of them are relieved instantly by a bowel movement

(Alvarez, 1919). Any student of biochemistry must admit that under those circumstances the symptoms cannot be due to a poison circulating in the blood because in that case relief would not follow immediately after removal of the source of the poison but later, when sufficient excretion had taken place to lower the concentration of the substance in the blood. To use a homely simile, one does not sober a drunken man immediately by taking his flask of whiskey away from him.

A search through the literature shows that several men in the past have commented on this typical sudden relief from symptoms and have recognized the impossibility of dragging in a chemical theory to explain it. Hertz (1909), Hewlett (1916) and Cabot (1915).

Donaldson (1922) shows quite conclusively that these views are correct. He took five normal men, who were in the habit of emptying their bowels once or twice every day, and for a control period of one week recorded their reaction times for sight, touch and hearing; their basal metabolic rates; fatigue reactions; blood urea; uric acid; sugar and non-protein nitrogen: blood viscosity; indicanuria; roentgen-ray findings in the gastro-intestinal tract; blood pressures and pulse rates. This control period was followed by another one of voluntary restraint of defecation lasting ninety hours, or nearly four days. No drugs were used. The diet was the same, mainly a liberal lacto-ovo-vegetarian one. In each case the typical symptoms were those of "autointoxication;" the breath became foul, the tongue coated, and the appetite impaired. Four complained of flatulence. One had periodic attacks of nausea. All of them developed pronounced mental sluggishness and loss of the power of attention. There was depression, restlessness, irritability and poor sleep, All developed headache. The reaction times were all increased. The basal metabolic rate rose to plus 18.1 per cent in one case, and averaged 6 per cent plus in the others. The blood sugar was increased. The blood urea, non-protein nitrogen and uric acid showed no change. The ergograph showed a more rapid onset of fatigue. The viscosity of the blood remained the same. There was no significant increase in indican. The barium meal showed that all the food remnants went into the colon and in every case the ileum remained clear.

At the close of the ninety hour period four of the men took enemas and the fifth had a large spontaneous bowel movement. They all were promptly relieved of their distress and within the hour they were back in the laboratory. One of them had a little headache for two hours, but the other four lost it immediately. The laboratory tests showed that the reaction times were back to normal. The ergographic performances were normal. The blood sugar dropped immediately to normal.

It is very interesting to note that the roentgen ray examination showed that the man who had failed to take an enema and who was entirely relieved of his symptoms by a bowel movement still had most of his colon, from the sigmoid to the mid-portion of the transverse, filled with feces. If his symptoms has been due to a chemical intoxication he could hardly have shown much improvement.

How are the symptoms produced? The next question is: If these symptoms are not produced by toxins, what are they due to? Something certainly makes them. The writer feels sure that *in this particular group of cases* they are caused by the mechanical distention and irritation of the rectum by the fecal masses. Perhaps the best proof of this assumption is to be found in the observation made by Pope (personal communication) and since confirmed by Donaldson, that the classical symptoms of "autointoxication" can all be produced simply by packing the rectum with cotton. The writer has observed this also with masses of barium and with cocoa butter suppositories.

Donaldson found with the cotton packing that the reaction times were prolonged and fatigue appeared promptly with exercise just as it did in the presence of feces. He found also that a similar packing of the rectum in a dog could raise the blood pressure 10 mm. of mercury in four minutes, and this level was maintained as long as the material was in the rectum. Years ago Zimmermann (1909) also found that the rectum is exceedingly sensitive to pressure. Changes of from 2 to 3 mm. of mercury were perceived by his subjects and a rise of from 20 to 60 mm. caused distress. We must remember also that many of the people who complain bitterly of autointoxication symptoms are unusually sensitive and keenly aware of what is going on in their bodies. It is this sensitiveness which distinguishes them from those who can go for a week quite unconscious of the fact that their bowels are crammed with feces.

There will be little difficulty in accepting these views when we remember how profoundly sensory impulses from our digestive tracts can influence our emotions, our mental processes, and our vasomotor balance. Thus the sleepiness or dulness which worries the patient with autointoxication is experienced by many people after dinner. The mental irritability and the inability to concentrate are experienced by many before dinner, when hunger pains are coming frequently; and the patient with a duodenal ulcer and an overly active stomach is not able to use his brain very much during his attacks.

The fact that one of the writer's patients with a jejunal fistula would often go to sleep when the intestine was made to contract actively on a

small balloon inserted through the fistula suggests that it is muscular activity and not the absorption of food which causes the drowsiness. Such instances and illustrations could be multiplied, all showing that nervous stimuli can arise in the digestive tract: stimuli which can profoundly influence the brain.

Many of the symptoms which are ascribed to auto-intoxication, such as the coat on the tongue, the bad breath and the "biliousness" can also be due simply to the plugging of the lower end of the canal which slows or stops the downward current and which perhaps produces ripples of reverse peristalsis. The food cannot go downward so the bowel tries to refuse it admittance or sends it back again. The writer has seen many instances in which the removal of the rectal plug has given immediate relief from regurgitation and belching. These reverse ripples deposit gastric and perhaps even intestinal contents on the back of the tongue, and in that way give rise to the coat on that organ and to the bad taste and the bad breath. (Alvarez, 1917.) The relief obtained by taking calomel can hardly be due to any action on the liver because the pharmacologists all agree that there is none, but is due apparently to a restoration of the normal downward currents.

It must be emphasized at this point that this explanation for the auto-intoxication syndrome applies only to those cases in which symptoms produced by constipation are relieved immediately after an evacuation of the bowels. Unfortunately most men who quote from the paper by Alvarez assume that he means that there is no such thing as chemical autointoxication, and that this mechanical explanation is all-sufficient. No such assumption was made or intended. It seems very probable that at times there is such a thing as an absorption of harmful toxins from the digestive tract, but that assumption can no longer be used to explain the production of symptoms in this particular, circumscribed group of cases. Fortunately for students of the subject, this group is so large that its exclusion greatly narrows the field for further investigation.

Very little absorption from the colon. There are other reasons for doubting whether there can be any absorption of toxins in the distal two-thirds of the colon. We know that feces begin to harden in the ascending colon and that thereafter they undergo practically no churning movements. Individual lumps tend to go forward through the remainder of the bowel like cars on a track. This can be shown not only with the roentgen-ray, but particularly by the study of fecal masses in which one can sometimes see sharp lines of cleavage between residues from two different meals. Very little absorption can be expected under

such circumstances; and in fact, many writers on the subject have very properly concluded that intestinal autointoxication is far more likely to take place during the course of *diarrheas.*

We know also that with the exception of water, there are few substances which are absorbed to any great extent from the colon. All those who have made careful studies with nutrient enemas have concluded that little besides some salt and sugar can be taken up; and some of that absorption may be dependent upon a regurgitation of the enema into the ileum. See Bywaters and Short (1913); Edsall and Miller (1902); Wynter (1902); Adler (1915). There is much evidence to show also that the mucous membrane of the colon is designed partly for the *excretion* of substances such as the heavy metals. Cannon (1912) has suggested that some absorption of toxic products might take place directly into the general circulation by way of the lower hemorrhoidal veins. The objections to that are that the feces are driest in that region, and that ordinarily they are held back in the upper rectum or sigmoid.

Many of the writers on autointoxication have recognized these objections to their views and have struggled to evade them. It will probably surprise many of the writers on this subject to learn that Bouchard felt that colonic stasis could do little harm, and that he even went so far as to say that with the drying out of the bacteria in the hard dry feces, "constipation ought to be regarded as a protection against autointoxication." He thought that poisoning was most likely to occur in diarrhea and particularly with dilatation of the stomach. We know now of course that the stomach rarely retains its contents long enough for any great amount of fermentation or absorption of toxins. Combe also admits that colonic stasis probably can have little effect on health, and that the absorption must be looked for elsewhere. Actually the roentgen-ray shows us in thousands of constipated people that the colon is practically the only place in which stagnation does take place.

There is little question but that stagnation in the lower ileum can do harm, and it is quite probable that regurgitation of colonic contents through the ileocecal valve produces symptoms in some cases (Jones, 1923).

Mutch, who has worked with Lane, believes that the terminal ileum is about the only place in which toxic substances can be formed and absorbed. The ability of the upper small bowel to absorb toxic excretory products was well shown in a case reported by Cecil and Cummings (1918) in which a uretero-intestinal anastomosis followed an injury to the abdomen. The patient was so severely poisoned by the absorption of urine that the kidney had to be removed.

Fecal impaction. There are a number of cases reported in which symptoms of disease were entirely relieved by an ileo-sigmoidostomy in spite of the fact, revealed by the roentgen-ray, that the colon was still filled with stagnating feces. (See Mutch.) The writer has seen one such case in which the steady advance of a deforming arthritis was stopped by an anastomosis of the terminal ileum into the mid-region of the transverse colon. This relieved an inveterate constipation and cured the arthritis, but subsequent roentgen-ray studies showed her enormous cecum still distended with stagnating feces.

Any busy physician knows many people who enjoy good health in spite of the fact that their bowels move but once a week. They must either produce little poison in the bowel or else they absorb none, or they are immune to what they do absorb. Similarly, the literature contains many records of men and women who have gone for months or years without a bowel movement and without the typical symptoms of autointoxication. Geib and Jones (1902) describe the case of a man who became constipated at eleven. During the next eighteen years he often went from three weeks to three months without a bowel movement. At the age of twenty-nine he went for five months and later for six months. Finally he went from June 18, 1900, to June 21, 1901 without a movement. During these periods of constipation he ate well and did a good day's work. He had much gas and some pain, and naturally his abdomen became greatly distended. After the year's stagnation he passed about eight gallons of feces. Toward the end of that period he had lost weight and had become weak. After his colon was cleaned out, he rapidly recovered. Harris (1913) reports the case of a man who went for fifty-three days with impaction, during which time he was free from headache and suffered very little except from gas. He worked hard about the farm and ate three meals a day. He seems to have had no symptoms of autointoxication and his temperature remained normal. His skin did not become pigmented. Toward the end of the period he lost weight. His indican was only slightly increased. His red blood count was practically normal in spite of the fact that he had a carcinoma of the colon. At the end of the period, some sixteen pounds of feces were removed. Such instances might be multiplied (see Pepper's *System of Medicine*, 1885, ii, 646) showing that the body defenses against such insults can be remarkably efficient.

There are instances reported, and the writer has seen some of them, in which a fever has dropped rapidly after the evacuation of the bowel. Some of those reported in the literature as due to copremia, on careful

study appear really to be cases of tuberculosis or some other febrile disease. In these cases the fever sometimes drops so suddenly after purgation that the question arises again: May it not have been kept up in some nervous way and not by a chemical mechanism? We know that a patient's fever can be increased by a too prolonged visit of a friend, and we know that it may drop very suddenly afterwards. We need more careful studies on this point, giving the exact time intervals between the evacuation of the bowel and the drop in the temperature.

HIGH INTESTINAL OBSTRUCTION. Many have felt that the finding by Whipple and others of an extremely toxic substance in the loops of bowel above obstructions made high up in the small intestine offers strong support to the theory of intestinal autointoxication. A little scrutiny of this work which will be found reviewed in articles by Whipple (1919), Ellis and others will show that such is not the case. The toxin of intestinal obstruction, which appears to be a proteose, is not formed in the colon at all, and is formed very slowly in the lower ileum. Dogs with closed loops of ileum or colon can live for long periods of time in perfect health. Furthermore, this toxin, when injected in large amounts into normal loops of intestine, does the animal no harm. Apparently it is absorbed from the mucous membrane only when it is formed there. There are many observations which make it seem very probable that its formation is not at all dependent on the action of bacteria. We have at present no reason for assuming that this substance is ever formed chronically in small amounts in such a way as to damage the individual.

Latent and sub-infection. We come now to a discussion of yet another possibility, and that is that many of the syndromes which are commonly ascribed to the passage of toxins through the intestinal wall may be due to the passage of actual living bacteria into the mesenteric glands and even into the blood stream. This point has been brought out particularly well by Adami and his students. For years, in spite of much evidence to the contrary, we have clung to the idea that the body tissues are normally sterile, and that the blood carries living organisms only in such grave diseases as malignant endocarditis, septicemia, typhoid fever, etc. It seems clear now that the intestinal bacteria are constantly passing through the mucous membrane, into the lymph spaces in the mesentery, into the glands, and into the blood. Most of these bacteria are destroyed rapidly as they pass to the root of the mesentery, but many of them secure a more or less permanent foothold in the various organs of the body and particularly in those places in which the tissue has been damaged or handicapped in some way.

Ford (1900), Ficker (1905), Bierotte and Machido (1910) and others have shown that the organs of many presumably healthy animals contain viable bacteria. These are not due to accidental contaminations because one group of organisms will be found in the pig, another in the rabbit and still another in the dog.

Recently David and McGill (1923) found living bacteria in over 50 per cent of the mesenteric glands of normal dogs. They were able to infect the urinary tracts in three out of eleven animals by putting large amounts of B. coli cultures into their intestines. A moderately prolonged constipation or diarrhea in the dogs was not associated with any increase in the number of organisms in the mesenteric glands, but an obstruction of the large bowel, or its traumatization, produced an increase.

Articles by Ravenel (1907), Hess (1910), Hilgermann (1905) and Ficker (1905) open up a large section of the literature on the passage of foreign bacteria like B. tuberculosis and B. prodigiosus through the bowel. These germs go through with remarkable ease and with great rapidity. It is interesting that Hilgermann, and Ravenel and Hammer (1911) found little passage through the mucous membrane of the colon as compared with the rapid passage through the mucous membrane of the upper bowel. It is suggestive also that such passage can be shown most easily in young animals and in children.

The work of Rosenow has shown that many troubles like myositis are due, not to toxins, but to the actual local growth of bacteria of low virulence. This is what Adami calls latent infection. He uses the term subinfection to explain the changes which may gradually take place in an organ that is continually having to destroy small numbers of bacteria. Eventually these tissues get worn out or damaged, and then chronic or acute infections supervene. Perhaps this is the sort of process which takes place in the development of cirrhosis of the liver.

Adami feels that it is much more rational to regard the evil effects of intestinal stasis in man as due, not to autointoxication, but to conditions favoring subinfection. He feels so strongly on this point that he says: "The term gastrointestinal autointoxication is pernicious and not to be employed by any self respecting member of our profession save for so limited a set of conditions that for ordinary purposes it may safely be wholly expunged from our medical vocabulary."

Now what lessons can be learned from the literature which we have just reviewed? In which direction do the sign-posts point for future research? In the first place, it seems clear that we must get the help of

biochemists. Without their exact methods we are likely to gather only misinformation. Then we must use fewer rabbits and more omnivorous animals like the rat. For some experiments we should use the cat and the dog whose digestive tracts are short and stout-walled, and more like ours. We must be surer that these animals are healthy to start with or else we will get into the position of those workers on encephalitis who did not know that the brains of half of their animals were already infected with a parasite, or the workers on typhoid who used animals already suffering from a chronic paratyphoid infection.

Animal experiments will have to be done with small doses of the suspected toxins given by mouth over long periods of time. We must study particularly the factors which favor or which hinder the passage of substances—solutions, colloids, foreign bodies and bacteria—through the mucous membrane. We know that our defensive mechanisms are wonderfully efficient most of the time. Why do they break down, and how can we restore their efficiency?

Some beginnings have been made on these problems. For instance, Dietrich (1922) has recently pointed out that although mice can ordinarily take large amounts of tetanus toxin by mouth without coming to any harm, they cannot do so if they are first given some bile or bile salts. Similar experiments have been done with rabbits showing that the giving of bile will render them susceptible to an alimentary tract infection with typhoid bacilli. (Besredka, 1922.) Other work has shown the importance of diet in modifying the resistance of the animal to infection. Thus K. F. Meyer (unpublished observations) has found that guinea pigs, which are normally immune to the effects of typhoid bacilli given by mouth, will become infected if they receive the germs while they are on a diet of bread and milk. Salant and Swanson (1918) have noticed that even when using such poisons as tartrates, a big difference in the resistance of the animals can be brought about by changes in their diet.

Starvation seems to favor the passage of bacteria through the intestinal mucosa, especially in rabbits (Ficker, 1905). In other animals it has been noticed that the germs are particularly likely to get into the lymphatics during the digestion of large meals rich in fat (Desoubry and Porcher, 1895). Anything which increases the secretion of mucus and opens up the beaker cells will facilitate the passage of bacteria (Booker, 1897). Dr. K. F. Meyer has found that when rabbits are purged they often developed a bacteremia. A good discussion of this subject of the penetrability of the intestinal wall to bacteria will be

found in an article by Beitzke (1910). The work of Macallum (1924) must be commented upon again because it must alter our conceptions about the passage of colloids through the intestinal mucosa. It seems clear that, although in some ways, and for a good deal of the time, the intestinal barrier is remarkably efficient, in other ways it can be very vulnerable, and it is that vulnerability which we must now study.

The recent work of Warren and Whipple (1923) is hopeful because it shows that experimental animals do not develop septicemias even when therapeutic doses of roentgen ray have destroyed the mucosa over large areas of the bowel wall. This would indicate that the second line of defense is also efficient.

Wassermann and Ficker (1922) have made some observations which suggest that certain substances, like those which when added in minute amounts to yeast cultures increase the growth many times, may occasionally get into the bowel and may enable otherwise harmless bacteria to produce substances highly toxic to the host.

We need to know more about the anerobes in the bowel and about the potentialities for evil of various symbiotic combinations of bacteria. More work needs to be done with Eck fistula animals and on men and women with definite insufficiency of the liver. Finally, much light may be thrown on the subject when competent statisticians show us what correlation there is between intestinal stasis and various diseases in large groups of men and women.

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SULFUR METABOLISM

HOWARD B. LEWIS

Department of Physiological Chemistry, University of Michigan

The literature dealing with the sulfur compounds which play a rôle in metabolism has become so vast that to attempt a comprehensive review in the limited space available is impossible. For this reason no claim to completeness is made for either discussion or bibliography. The present review concerns itself with a series of problems of sulfur metabolism which seem essential, particularly in the light of more recent advances.

I. THE SULFUR CONTENT OF THE DIET. *a. Non-protein sulfur compounds:* Of the various forms of sulfur which are concerned in metabolism, the greater part enters the body in organic combination and chiefly as protein sulfur. Few analyses of the inorganic sulfate content of our more important foods exist, since most of the data on foods have been obtained by analyses of the ash and, as such, include in part, at least, sulfur present in combination in the protein molecule. Such data as are available and are not open to the criticism above cited, indicate that, in animal foods, at least, the sulfate ion is present in very minute quantities. Denis (37) has reported from 0.5 to 4.0 mgm. of sulfur as inorganic sulfate per 100 cc. of blood (man and the more common domestic animals), figures which are of the same order of magnitude as the few others reported for sulfate sulfur in mammalian blood (36), (64). Macallum (107) in his studies on invertebrates, found 2.4 and 4.7 mgm. of sulfur as sulfates per 100 cc. in the blood of the horse shoe crab (Limulus polyphemus) and the lobster (Homarus americanus). The average inorganic sulfur content of four muscles of the cat (diaphragm, extensor, sartorius, soleus) as determined by Lee, Scott and Colvin (92) was 69 mgm. per 100 grams of fresh muscle. Koch and Koch (86) found 7.7 mgm. of inorganic sulfur per 100 grams of fresh brain tissue in the white rat at 210 days of age.

A certain amount of sulfur in organic combination other than protein, also exists in animal foods as sulfo-lipoids, the sulfatide of Levene (93) and the "phosphatide-cerebroside-sulfatide" of Koch (85). Koch and Koch (86) determined the lipoid sulfur (0.198 per cent) in the brain of

the white rat at 210 days. The muscles of the cat contain 0.0121 per cent of lipoid sulfur (92), slightly less than 6 per cent of the total sulfur of the muscles. The metabolic history and exact function of these sulfolipoids are not known.

b. Sulfur in the protein molecule: Although the nitrogen content of proteins of different types does not vary markedly from an average figure of 16 per cent, the same proteins exhibit marked variations in their sulfur content. While the ratio between nitrogen and sulfur in proteins is approximately 16:1, many notable exceptions may be cited. Osborne (127), to whose studies of proteins much of our information is due, noted a range of from 0.200 per cent (vicilin, the globulin of the pea and certain other legumes) to 1.930 per cent (serum albumin). The small amount of sulfur in gelatin and collagen has frequently been ascribed to the presence of other proteins as impurities, since it is exceedingly difficult to remove all material other than collagen from the tissue, prior to the preparation of pure gelatin. Van Name (122) however, found 0.25 per cent of sulfur in a very pure sample of gelatin prepared from tendon. Mörner (114) observed that commercial gelatins contained varying amounts of sulfates which could be removed by soaking the gelatin in water. Gelatin, which was washed from 1 to 10 days with 0.1 per cent potassium hydroxide and 4 to 6 days longer with water or weak acetic acid, still contained 0.20 per cent sulfur. That the low sulfur content was not due to the action of the alkali was shown by the fact that gelatins which were in contact with the alkali for only one day contained no more sulfur than those which were washed with alkali for 10 days. Moreover, a sample washed 12 days with distilled water contained 0.22 per cent sulfur. The constancy of the sulfur content of the gelatins purified by Mörner would seemingly indicate that sulfur is a definite constituent of the molecule in gelatin and is not present as an impurity. Dakin (33), who has recently investigated anew the amino-acid content of gelatin, concluded that "a significant amount of unidentified sulfur compounds has yet to be accounted for" in gelatin. The keratins on the other hand contain more sulfur than any other group of proteins, the sulfur content ranging from 2.22 per cent in tortoise shell to 5.7 per cent in human hair (26), (41).

Cystine, discovered by Wollaston (174) in 1810 in a urinary calculus, and isolated as a product of tryptic digestion of fibrin by Kulz (89) in in 1890 and by Emmerling (45) in 1894 from the hydrolysis products of horn, is probably the main sulfur-containing constituent of the protein molecule (43), (116). Much evidence, however, which points to the

existence of sulfur compounds other than cystine has accumulated and is well summarized by Johnson (81). The difference between proteins in the content of loosely bound sulfur appears particularly significant. When proteins are boiled with alkali, varying amounts of sulfur are split off as sulfide. This sulfur which is readily yielded on treatment with alkali is known as loosely bound sulfur. The following table condensed from Osborne (127) shows the ratio between loosely bound sulfur and total sulfur in some of the more important proteins:

TABLE 1

Ratio between loosely bound sulfur and total sulfur

	PER CENT OF LOOSELY BOUND SULFUR
Seralbumin	66.0
Gliadin	60.0
Oxyhemoglobin (dog)	59.0
Serglobulin (horse)	57.0
Edestin	40.0
Zein	35.0
Fibrin	34.0
Ovalbumin	30.0
Casein	13.0

It will be noted that the percentage of loosely combined sulfur runs roughly parallel to the cystine which can be isolated from the proteins. Thus in serum albumin with about two-thirds of its sulfur loosely combined, the probable amount of cystine according to Harris (70) accounts for 89 per cent of the total sulfur present, while in ovalbumin, only 14 per cent of the total sulfur present may be accounted for as cystine. Casein, from which traces only of cystine have been isolated, yielded the lowest proportion, 13 per cent, of its sulfur on boiling with alkali. Some exceptions to the above must, however, be made. Thus fibrin which contains 3.5 per cent of cystine as determined colorimetrically (50) splits off much less of its sulfur by the action of alkali than does gliadin with a lower cystine content (50).

The work of Mörner (115) is also of importance in this connection. In his studies of the oxidation of proteins with nitric acid, he was able to isolate from the products of oxidation methyl sulfonic acid (CH_3SO_3H) as the barium salt in varying amounts. The yield of the barium methyl sulfonate bore no relation to the cystine content of the protein oxidized (table 2). Casein from which cystine has never been satis-

factorily isolated yielded more of the sulfonic acid derivative than did wool, the most readily available source of cystine. Control experiments in which the oxidation of cystine by nitric acid was studied, demonstrated that this substance could not be the mother substance of the methylsulfonic acid obtained. The work of Mörner affords further evidence of the existence of another sulfur-containing complex in the protein molecule.

Mueller (117) in 1921 isolated from the products of acid hydrolysis of casein a sulfur containing amino-acid to which the formula $C_5H_{11}SNO_2$ was later given (118). The new amino-acid was also obtained from egg albumin, edestin, wool and gelatin by acid hydrolysis; from casein by alkaline hydrolysis; and from a commercial protein hydrolysate pre-

TABLE 2

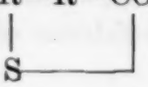
The relation between the cystine content of proteins and the yield of barium methyl sulfonate on oxidation

	GRAMS PER KGM. MATERIAL	
	Barium methyl sulfonate isolated	Cystine present
Serum protein	3.1	12.0
Egg white	5.8	3.0
Casein	5.5	0.2
Ovomucoid	12.4	
Hemoglobin	3.1	3.1
Keratin (sheep wool)	1.8	73–125
Gelatin	1.4	0.0

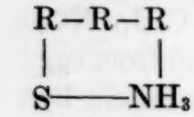
pared by enzymes. Inasmuch as no larger yield was obtained from wool, high in its content of cystine, than from casein, in which little, if any, cystine is present, the mother substance of the new acid, if it is to be regarded as the result of a secondary reaction, can hardly be considered to be cystine. Although the new acid has an empirical formula corresponding to ethylcysteine, its properties are quite different from those of this substance. The sulfur of the new amino-acid has been shown to be readily oxidized to sulfates by the animal organism (119). Further investigations of this amino-acid should aid greatly in the solution of the problem of the sulfur of the protein molecule.

Johnson (81) has postulated the existence of thiopolypeptides containing thioamide groups derived from thio-acids. Harris (69) in his studies on denaturation of proteins has also discussed the possibility

of the existence of thiopolypeptides of the type R−CS.NH−R. A linkage of the −COS type in which −SH replaces NH_2 in the normal peptide linkage is also suggested, R−CO.S−R. Other possibilities suggested which merit further study are, condensation between non-adjacent sulfhydryl and carboxyl, R−R−CO and the replacement of



carboxyl by sulfhydryl functioning acidically, R−S.NH_3−R or

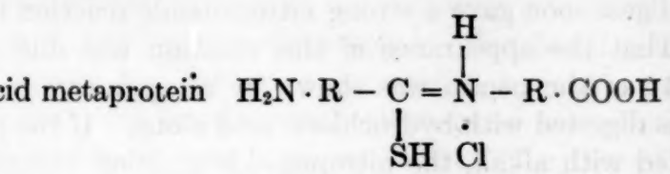


Despite the fact that for many years cystine has been recognized as an important unit among the products of protein hydrolysis, definite proof as to whether the disulfide, cystine, or its reduction product, cysteine, actually exists in the protein molecule is lacking. The presence of cysteine in tissues and proteins was suggested by Arnold (16) on the basis of the nitroprusside reaction (15), (72), (116). In the presence of ammonium hydroxide, sodium nitroprusside reacts with cysteine (and other compounds which contain the mercapto group) to give an intense purple red color, which fades on standing. The color reaction is also given in the presence of sodium or potassium hydroxide, but is then less specific, since creatinine also reacts, under these conditions (Weyl's test). Abderhalden and Wertheimer (8) have shown that fading does not occur in the presence of potassium cyanide, probably due to the inhibition of the oxidation of the cysteine to cystine by the cyanide. This reaction, the importance of which was earlier pointed out by Buffa (27) and Heffter (72), is characteristic of substances which contain the mercapto group, −SH, e.g., cysteine, *a*-thiolactic acid, thioglycollic acid, ethyl mercaptan, etc. Heffter obtained a weakly positive reaction with ovalbumin which became more marked after heat coagulation, while with serum protein, fibrin, ovoglobulin and Witte's peptone, the reaction was negative. Arnold (15) reported positive reactions with the crystalline lens of the eye and with peptic digests of egg white. From extracts of the lens, the protein which was precipitated by neutral salts, alcohol or phosphotungstic acid, showed a positive test. After one hour's digestion of egg white with pepsin-hydrochloric acid a very marked reaction was obtained.

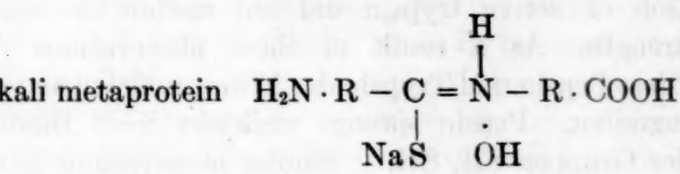
Although cysteine had not actually been isolated from protein, the attention of students of protein chemistry was again focused on the possible occurrence of cysteine by the brilliant work of Hopkins (77)

in 1921. The occurrence of a dipeptide of cysteine and glutamic acid, for which the name glutathione was suggested, was demonstrated in animal tissues as well as in yeast. To the presence of this substance was attributed the nitroprusside-ammonia reaction of tissues. Later work by Harris (69) and Abderhalden and Wertheimer (12) indicates that if cysteine does not actually exist as such in native proteins, it may be easily formed as result of such relatively simple reactions as those involved in heat or alcohol coagulation, formation of alkali or acid metaprotein, or in the early stages of digestion by pepsin. Harris suggests that acid metaprotein, alkali metaprotein and coagulated protein may be represented by the following formulae all containing reactive $-SH$ groups.

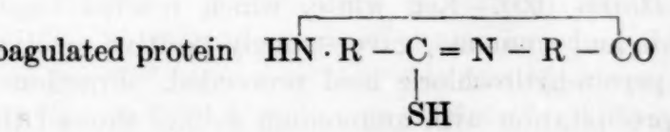
Acid metaprotein



Alkali metaprotein



Coagulated protein



The validity of this conception, however, is largely dependent upon the specificity of the nitroprusside color test. Until it has been definitely proven that no groups which will react with sodium nitroprusside and ammonia, other than the mercapto groups, exist in the protein molecule or until cysteine shall have been actually isolated from the products of protein hydrolysis, the presence of cysteine in the protein molecule must be regarded as probable, but not proven.

In addition to the sulfur present in organic combination in the protein molecule, sulfur of a different type exists in the prosthetic groups of the glycoproteins, as glucothionic acid or mucoitin and chrondroitin sulfuric acids (94). These acids are unique among the known products of tissue anabolism in that they contain sulfuric acid (5.3 per cent sulfur), in combination with carbohydrate, presumably in ester linkage. On

account of the difficulties in their preparation and purification, we do not possess as exact knowledge of the behavior of these conjugated sulfuric acids in the digestive tract or in the tissues, as in the case of the nucleic acids, the prosthetic groups of the other great class of conjugated proteins, nucleoproteins. According to Neuberg and Rubin (125), putrefactive organisms readily split off sulfuric acid from chondroitin-sulfuric acid so that it is not unlikely that a significant part of the sulfate sulfur of the urine may originate from the bacterial decomposition of the mucous secretions of the alimentary canal.

2. THE ACTION OF THE DIGESTIVE SECRETIONS AND THE INTESTINAL FLORA ON SULFUR COMPOUNDS. Abderhalden and Wertheimer (12) found that when frog muscle was digested with pepsin and hydrochloric acid, the digest soon gave a strong nitroprusside reaction for mercapto groups. That the appearance of this reaction was due primarily to the activity of the pepsin was shown by comparative tests in which muscle was digested with hydrochloric acid alone. If the peptic digest were treated with alkali, the nitroprusside reaction became weak and the addition of active trypsin did not restore the reaction to its former strength. As a result of these observations Abderhalden suggests "dass Pepsin und Trypsin das Eiweissmolekul an verscheidenen Stellen angreifen. Pepsin sprengt vielleicht S−S Bindungen unter Bildung der Gruppen SH, SH." Similar observations have also been made by Harris (69). Egg white, which reacted negatively with nitroprusside and ammonia, gave strongly positive reactions as digestion with pepsin-hydrochloric acid proceeded. Fractionation of the digest by precipitation with ammonium sulfate showed that the proteoses, peptones and polypeptides, all contained a group reactive to nitroprusside. Under anerobic conditions, the nitroprusside reaction given by the digest persisted for several months, but in the presence of air even in an acid solution, the reactivity disappeared in a few hours, and even more rapidly in alkaline solution. During tryptic hydrolysis of egg white, the nitroprusside reaction was not observed at any stage of the hydrolysis. This might have been due to the fact that oxidation of mercapto groups is favored by an alkaline reaction, a condition necessary for effective digestion by trypsin. No studies of the sulfur (and cystine) content of the products formed at different stages of peptic, tryptic and ereptic digestion of sulfur-rich proteins have been made in which the newer analytical methods for proteins and amino-acids have been employed. Such an investigation should throw considerable light on the problem under discussion.

The hydrogen sulfide, methyl and ethyl mercaptans formed in the intestine by microörganisms and either absorbed or eliminated in the intestinal gases are known to originate mainly from protein sulfur (74). Wohlgemuth (171) was able to prove that cystine was the source of the hydrogen sulfide, methyl mercaptan and ethyl sulfide produced in putrefaction experiments and suggested that from the activity of the intestinal flora the ethyl sulfide of the urine of dogs (13) originated. The power of microörganisms to form hydrogen sulfide from a wide variety of sulfur compounds, (cystine, elementary sulfur, thiourea, sulfites, thiosulfates, but not taurine) is well known (160), (161).

The fate of hydrogen sulfide absorbed from the intestinal tract has been of especial interest in view of the well-known toxicity of this gas (113). Haggard (66) has recently concluded that blood plasma in the presence of oxygen has the power to oxidize hydrogen sulfide very rapidly, and that in normal animals there is no combination of the gas with hemoglobin to form sulfhemoglobin. The rate of oxidation of hydrogen sulfide in the blood was such that in a relatively short time many times the lethal dose of sulfides could be injected intravenously without any toxic effect. This rapidity of oxidation explains the comparatively slight toxic properties exhibited by the sulfides absorbed from the intestinal tract. The sulfur of mercaptans is oxidized in part in the animal organism and eliminated as sulfates (156) but little is known concerning the velocity or site of the oxidation.

The reported elimination of a considerable part of elementary sulfur fed to man or dogs as urinary sulfate or sulfites (71), (87) is probably to be ascribed to conversion of the sulfur to hydrogen sulfide by the intestinal microflora and absorption of this with subsequent oxidation to sulfates or sulfites.

Taurocholic acid is less stable than glycocholic and is so completely decomposed that it does not appear in the feces of adults. In the meconium, however, where no marked putrefaction occurs, the presence of taurocholic acid may be detected in the feces.

3. Forms of anabolized sulfur in the organism. *a. Cystine.* The importance of adequate amounts of cystine to insure normal growth of young white rats has been demonstrated by Osborne and Mendel (128), and later by others (3), (80). Abderhalden (5) fed diets composed of simple chemical substances (glycerol, fatty acids, amino-acids, etc.) and observed that lack of cystine in such diets resulted in failure of nutrition with symptoms of alimentary dystrophy. Maintenance of adult white mice is impossible without the presence of cystine (56).

Lewis (96) in studies on dogs maintained on low protein diets, has reported that the addition of cystine to the diet diminished the loss of nitrogen and favorably influenced the nitrogen balance. Serum albumin, a protein rich in cystine, was more efficient as a source of protein under conditions of low protein intake than was casein, a protein low in cystine content, although casein supplemented by the addition of cystine was as efficient for the maintenance of nitrogenous equilibrium as was serum albumin (97). Since neither glycine nor tyrosine when added to the diet influenced favorably the nitrogen balance, these results were interpreted as an indication of a specific demand for cystine by the tissues for maintenance as well as growth. This cystine cannot be synthesized by the tissues but must be furnished in adequate amounts in the diet. Neither elementary sulfur (56) nor sulfates (35) are able to serve as a source of sulfur (and cystine) in supplying the requirements of white mice or rats. The supplementary value of "protein-free milk" when added to the rations of white rats on a 10 per cent plane of protein (milk) intake has been attributed to cystine or some other organic sulfur compound,[1] which may be transformed into cystine (159).

Experiments (2), (21), (47), (54), (176), (177) which indicate a special relation of cystine to the growth of the sulfur—(and cystine) rich epidermal tissues (hair, wool, etc.), are interesting, but further work under more carefully controlled conditions is necessary before the specific rôle of cystine in the growth of hair can be accepted without qualification.

Interesting studies have recently appeared from the laboratory of Thomas in Berlin in which the utilization of cysteine for the detoxication of brombenzene, discovered independently by Baumann and Preusse (18) and by Jaffé (79), is applied to the study of the problem of the supply of cystine available in the animal organism. When dogs on a protein-free or very low protein diet were fed brombenzene, no synthesis of bromphenylmercapturic acid occurred (163), (175). Since in these experiments any cysteine available for detoxication of the brombenzene must have been liberated in the tissues from the breakdown of the body

[1] Osborne and Mendel (Journ. Biol. Chem., 1924, lix, 339) have recently discussed the supplementary value of "protein-free milk" and conclude that "Obviously, therefore, no great part of the nitrogen of the 'protein-free-milk' belongs to any one of the essential amino-acids; and consequently, it can not supplement to an important degree any amino-acid deficiency of the proteins of the diet unless the latter are fed in very small proportion."

protein and since the benzene compound entered the body from the alimentary canal, it seemed possible that the brombenzene never came in contact in the proper organ with the cystine or cysteine necessary for the synthesis. However, when cystine was injected (83) into animals maintained on these diets, and the brombenzene fed, an abundant synthesis of bromphenylmercapturic acid occurred, thus proving that the paths of entry into the organism of these two components of the detoxication product need not necessarily be the same. From these experiments, it is argued that the reactions of the catabolism of body protein do not result in the formation of cystine in the same way as do the processes of autolysis or of digestion in the alimentary canal. In these studies it was observed that after brombenzene feeding, even in the absence of bromphenylmercapturic acid synthesis, the urinary elimination of organic ("neutral") sulfur was increased. It is unfortunate that this phase of the problem was not studied further since investigation of the chemical nature of this increased organic sulfur should have furnished a clue to the chemical reactions involved. These findings have been recently confirmed and extended by Sherwin and his associates (120) who also noted that under experimental conditions similar to those discussed above, neither sodium sulfate, taurine, calcium sulfate, ethyl mercaptan nor sodium thiocyanate could be utilized for the elaboration of the cysteine necessary for the detoxication of brombenzene, even when nitrogen for the amino groups was added to the diet either as inorganic nitrogen, ammonium acetate or organic nitrogen, gelatin. The inability of the human organism, either normal or cystinuric, to carry out the synthesis of bromphenylmercapturic acid after the ingestion of brombenzene (111) cannot be explained on the basis of our present knowledge. No increase in the "neutral" sulfur of the urine after brombenzene was noted.

It has been suggested by Harding and Young (67) that cystine is a precursor of creatine in the growing dog, through the intermediate stages of taurine and aminoethyl alcohol, followed by methylation, combination with urea and oxidation. In view of the fact that creatine is determined as creatinine after dehydration in the presence of acids and that many of the reactions by which creatinine is usually identified are also given by cysteine and mercapto derivatives, it seems possible that the variations of the creatine content of the urine observed may have been due to the presence in the urine of mercapto or other derivatives of cystine. Increased excretion of creatine by the pig has been observed after the ingestion of cystine (65). The increases were, how-

ever, considered to be due to the acidosis resulting from the oxidation of the cystine sulfur to sulfuric acid.

b. Taurine. Noteworthy among the substances which owe their origin to cystine in the organism is taurine, aminoethylsulfonic acid. This substance, which is unique among the products of the activity of the living cells, a sulfonic acid derivative, occurs in the bile in conjugation with cholic acid, and also free in considerable quantity in the tissues of many lower forms (notably the abalone) (73), (84), (110), (144). The content of taurocholic acid in bile varies greatly with the species, being greatest in the bile of carnivora (dog), although found also in the bile of certain herbivorous animals (ox, sheep, goat) and in human bile. The precursor of this taurine is usually considered to be cystine and inasmuch as we know of no difference in the amounts of cystine available for anabolic purposes in different species, it is difficult to understand why the bile of certain animals (rabbit) should be almost entirely lacking in taurocholic acid.

Taurine appears to be present in amounts in excess of those needed to combine with the cholic acid of normal metabolism, since the amount of taurocholic acid secreted in the bile is not increased by the administration of taurine alone (51), but when cholic acid or its salts are administered simultaneously with the taurine a marked increase in the excretion of taurocholic acid may result (51), (168). The amount of taurocholic acid secreted is presumably limited by the available supply of taurine, since the feeding of cholic acid during fasting gives a minimal output of bile acids (51) and may even result in the excretion of glycocholic acid in a species (dog) (168) in which this form of conjugated bile acid is not normally present (142). In the latter case, the excretion of glycocholic acid is reported to decrease if taurine is also fed.

Friedman (53) first demonstrated in vitro the synthesis of taurine from cystine. Von Bergmann (20) noted that the output of taurocholic acid was increased in dogs when cystine and sodium cholate were fed together, and concluded that the synthesis of taurine from cystine, suggested by Friedmann, actually occurred in the living organism, a conclusion which was supported by the work of Wohlgemuth (171) and of Foster, Hooper and Whipple (52). Gibson (58) studied the problem of the relation of cystine to taurine by a different experimental method. He attempted to deplete the available supply of cystine in the body by the injection of brombenzene for the detoxication of which cysteine is utilized. The alcohol soluble sulfur of the bile of cats poisoned with brombenzene was markedly decreased, which was inter-

preted as an indication of a shortage of the precursor of taurine, cystine, in the organism. Blum (23) also reported that in dogs with biliary fistulas, the production of an experimental cystinuria (presumably by brombenzene, although not definitely stated) caused an almost complete disappearance of taurine from the bile.

These attempts to study the influence of an experimentally produced cystinuria on the synthesis of taurine may be compared with similar studies on cystinuria in man. Marowsky (108) studied a case of cystinuria with involvement of the liver resulting in a complete chronic acholia and suggested that the kidneys acted vicariously in the excretion in the urine of cystine, which would normally have been converted to taurine and excreted in the bile. (Garrod (55) estimates that 30 per cent of the cystine formed in the body from the breakdown of body proteins is utilized for the formation of taurine.) Simon and Campbell (152) believed that in the cystinuric individual taurocholic acid may not be formed at all, but a recent analysis of gall bladder bile, obtained at autopsy, from a case of cystinuria (46) showed a content of taurocholic acid only slightly below normal.

Although it may be regarded as rather definitely proven that cystine is the substance from which the combined taurine as taurocholic acid originates, the mechanism of the reaction by which the cystine is converted is not known. Friedman's synthesis of taurine from cystine (53) in which cysteinic acid was formed as an intermediary product, although possible in vitro, may not occur in the living organism. There is some evidence (22) which indicates that cystine may combine with cholic acid before oxidation of its sulfur and the conjugated product then be oxidized to taurocholic acid. If this were the reaction for the formation of taurocholic acid, free taurine might never be present in the body. In opposition to this theory is the fact of the occurrence of free taurine in other tissues than the liver, especially in invertebrate muscle, as already discussed.

The fate of ingested taurine is not definitely known. The ingestion of taurocholic acid by the dog does not increase the sulfur elimination in the urine (141) nor is there an appreciable excretion of bile acids through the kidneys even in icterus (143). Evidently the urine is not a channel for the elimination of bile acids.

It has been suggested that taurine may be an intermediary product in the oxidation of cystine to sulfates and that the conjugation with cholic acid represents a product deviated from its normal path of catabolism for special purposes. The reversal of the reaction, whereby

taurine is supposed to be formed, is not accomplished by the organism (120), even under the stress of an urgent demand for cysteine. The fact that taurine is not readily oxidized when ingested would indicate that its formation is not a process in the main reaction involved in the oxidation of cystine. Taurine which was observed by Salkowski (134) to be toxic for rabbits, is oxidized to sulfates to a considerable extent by this species when ingested orally but not when injected subcutaneously (135). In man and in the dog, the administration of taurine results in a marked increase of the unoxidized ("neutral") sulfur of the urine and there is little evidence of any oxidation of the sulfur (135), (141). According to Salkowski, after the ingestion of taurine, taurocarbamic acid appears in the urine of man and the dog (135), but not in the urine of rabbits (136). Schmidt, however, while demonstrating in man that taurine increased the neutral sulfur of the urine (139) was unable to find any evidence of the existence of taurocarbamic acid in the urine.

It is probable that the taurocarbamic acid observed by Salkowski was formed after the secretion of the urine. Dakin (31) has shown that a similar reaction may account for the presence of the uramino derivatives of tyrosine and phenylalanine in the urine after the administration of these amino acids to cats. The failure of taurine to be oxidized in the animal organism is in harmony with the resistance of the sulfonic acid group in other compounds to oxidative processes of the body discussed in a later part of this paper. The reported oxidation of the sulfur after oral administration of taurine to the rabbit should be reinvestigated, with especial consideration of the possible action of the intestinal flora in the reduction of the taurine to sulfites or thiosulfates (125), (126).

c. Thiocyanates: The presence of a substance in the saliva which gave a scarlet coloration with ferric nitrate or sulfate was first discovered by Treviranus (165) in 1814. Since that time much controversy has arisen as to the occurrence and significance of the thiocyanates, not only in saliva but in urine and other body fluids. The older literature is well summarized by Kahn (82). The production of thiocyanates is usually considered to be a defensive process whereby the toxic CN radicals are converted into the less toxic SCN compounds. Gies and his collaborators (59), (60) observed an increased excretion of thiocyanate in dogs after ingestion of nitriles and certain amino-acids, glycine, alanine and leucine, and believed that normally the thiocyanate is dependent on the production of cyanogen compounds by the oxidation of certain groups in the protein molecule (endogenous origin). Dezani

(38), on the other hand, was unable to demonstrate an origin of thiocyanates from guanine, creatinine, asparagin, alanine, glycine or aspartic acid in the rabbit and reached the conclusion that the normal excretion of thiocyanates was exogenous in its origin. The results obtained after the ingestion of cystine by normal or pellagrous subjects by Bodansky and Levy (24) failed to demonstrate any marked influence of cystine as a source of sulfur on the excretion of thiocyanates, although the authors state that "the detoxification of potassium cyanide occurs more rapidly in the human organism when the administration of the cyanide is preceded by the ingestion of cystine." The results of Dezani (39) do not support the theory of Cavazzani of a physiological function of thiocyanates in the protection of the gastric blood vessels from corrosion by the hydrochloric acid of the gastric juice.

4. THE CATABOLISM OF CYSTINE AND RELATED SULFUR COMPOUNDS: Despite the fact that it has been recognized for many years that cystine ingested by the animal organism is almost completely oxidized to sulfuric acid and its sulfur rapidly eliminated as sulfate sulfur by the kidneys (172), little is known of the intermediate reactions by which the oxidation to the final end product, sulfate sulfur, is accomplished. It is difficult to produce an appreciable cystinuria by *feeding* cystine (22), (133), although after intravenous injection of moderate amounts into dogs, a considerable proportion of the injected cystine was observed by Blum (22) to appear in the urine within a relatively short time. Blum also noted that cystinuria occurred only after injection of the cystine into the peripheral circulation (jugular or crural vein) and was not observed when the path of injection was through the mesenteric venous circulation. It was suggested that this difference might be due to a specific influence of the liver on cystine catabolism, possibly through retention of the cystine in the liver tissue for the synthesis of taurine. The sulfur of cysteine is readily oxidized (32), (61), (150) in the body, as is also the sulfur of dipeptides containing cystine (6).

That the formation of cysteine is the first step in the catabolism of cystine seems to be definitely proven by recent studies in which complete oxidation of the cystine molecule was prevented by blocking the amino groups, thereby preventing deamination (100), (101), (102), (150), (151). This idea is in accord with the current theory (77) that the reversible reaction, cystine $\rightleftarrows$ cysteine, is an important factor in the control of tissue oxidation. Dakin (32) has demonstrated that cysteine gives rise to extra glucose in phlorhizinized dogs. Lusk (106) believed

that this conversion of cysteine to glucose might be accomplished by an oxidative desulfurization whereby serine and hydrogen sulfide are formed since serine has been shown to be converted almost quantitatively to glucose. The cleavage of hydrogen sulfide and its oxidation to sulfates can, however, hardly be regarded as the main reactions involved in the oxidation of the sulfur of cystine under normal conditions.

The point of attack in the oxidation of the sulfur of the cystine molecule is not known with certainty. If the amino groups of cystine were blocked by some organic radical, so that the resultant derivative could not be deaminized (e.g., phenyluraminocystine, dibenzoylcystine), the oxidation of the sulfur did not occur normally (100), (102). Some oxidation of the sulfur to sulfates occurred after feeding, but no oxidation was noted after subcutaneous injection. This was considered to indicate a close relationship between the oxidation of the mercapto groups and the process of deamination. Similar studies of the oxidation of cystine or cysteine derivatives in which the amino, mercapto or carboxyl groups were blocked led Sherwin and his collaborators (150) to conclude that when both the amino and mercapto groups of cysteine were blocked, oxidation was much decreased and that in the oxidation of cystine the attack was most effective at the amino position and least effective (possibly not at all) at the carboxyl group. The full details of these interesting studies are not yet available. From the preliminary reports, however, it would appear that the compounds were administered orally. Under these conditions changes produced by microörganisms in the intestine would undoubtedly result in some cleavage of hydrogen sulfide or mercaptan and these by their oxidation would make it appear that the sulfur of the compound fed had been oxidized by the tissues. The ease with which many types of sulfur compounds are attacked by microörganisms and the fact that although the sulfur of phenyluraminocystine is not oxidized when administered parenterally, a part of its sulfur appears in the urine in oxidized form after oral administration makes such an explanation appear probable.

The oxidation of sulfur in compounds other than cystine or proteins has been of particular interest in view of the therapeutic use of sulfur compounds (sulfonal, etc.). The sulfur of mercapto compounds (ethyl mercaptan, thioglycollic acid, etc.) is oxidized readily (98). The sulfur of organic sulfides (C-S-C) is oxidized with difficulty, if at all (98). Bivalent sulfur replacing bivalent oxygen as in thiourea (87), (91), (109), (138) or the thiopyrimidines (158) is not attacked. In this

connection, it is of interest to note that after the administration of the thiourea to rabbits or dogs, a volatile sulfide (ethyl sulfide) was eliminated in the expired air (129). The partially oxidized sulfur of sulfonic acids and sulfones is resistant to further oxidation in the organism (17), (141), (155). Reference should also be made to the previous discussion of the oxidation of taurine in the animal organism. To summarize, it appears that of the various types of sulfur linkages investigated, only mercapto groups or such others as may be hydrolyzed (thiourethane (154)) or reduced (cystine) to form this group are readily oxidized in the organism. An exception to the above statement must be made, however, in the case of mercapto groups attached directly to the benzene nucleus as in thiophenol or thiocresol (99), which resist oxidation.

5. THE SULFUR EXCRETION IN THE URINE: *a. The ratio of urinary nitrogen to urinary sulfur.* It has often been suggested that a study of the ratio of the excretion of sulfur to that of nitrogen should give valuable evidence concerning the rate of metabolism of the protein molecule. Von Wendt (169) has maintained that a true picture of protein catabolism cannot be obtained from a study of nitrogen excretion alone but only by a study of the combined nitrogen and sulfur excretions. If the daily sulfur output were multiplied by the factor 9.3, the ratio of nitrogen to sulfur of a fasting experiment, the figures obtained corresponded well with the figures obtained by direct determination of nitrogen in the urine. It should be noted, however, that the ratio used, 9.3, is much lower than the fasting N:S ratio obtained by other investigators, 13–16 (19), (95). In general it has been observed that the maximal elimination of sulfur after ingestion of protein food either precedes (28), (42), (169) or coincides (149) with the maximal nitrogen elimination. However, after the ingestion of cystine or cysteine by normal (141) or phlorhizinized (32) dogs, the elimination of nitrogen precedes that of sulfur. From the data obtained after the ingestion of protein food, it has been argued that the sulfur-rich moiety of the protein molecule is split and oxidized first by the organism while the sulfur-poor fraction is either retained or oxidized more slowly. The retention of a sulfur-poor fraction could not be demonstrated by the experiments of Tsuji (166). For an excellent summary of the literature, reference may be made to Cathcart's monograph on protein metabolism (29).

The output of sulfur in relation to nitrogen during prolonged fasting gradually increases with a resultant slight decrease in the value of the

N:S ratio (19), (95). In view of the importance of an adequate supply of cystine for tissue metabolism, it might be anticipated that the organism would conserve its sulfur compounds during fasting and that the N:S ratio would rise. This does not appear to occur. After a prolonged fast, the ingestion of large amounts of meat by a dog (95) was followed by a retention of both nitrogen and sulfur as was to be expected, but the retention of sulfur far exceeded that of nitrogen. On some days N:S ratios as high as 23 to 26 were observed. This would seem to indicate either a retention of a sulfur-rich protein in the period of recovery from inanition or a much more striking lag in sulfur elimination than is usually observed. A study of the sulfur content of various muscles and organs of the body after fasting and after the feeding of sulfur-rich proteins is in progress in the author's laboratory and should aid in the solution of this interesting problem.

Studies of the N:S ratio have also been made in connection with the problem of the significance of the nitrogen-sparing action of ammonium salts. It was argued that if the retention of nitrogen observed after the ingestion of various non-protein nitrogenous compounds were the result of a true protein-sparing, the elimination of urinary sulfur would be diminished, as well as the urinary nitrogen. Rosenberg (132) fed carbohydrate-rich diets to which ammonium salts were added. The carbohydrate-rich food itself so depressed the sulfur elimination that it was impossible to determine the effect of the addition of ammonium salts. His experiments, however, do demonstrate that cystine is more effectively protected by carbohydrates than are the other constituents of the protein molecule. Gesler (57) studied the effect of the feeding of ammonium salts to fasting dogs. Although in two experiments there occurred a considerable retention of nitrogen as compared with the fasting control periods (equivalent to about one-third of the ammonium citrate fed) the sulfur elimination was constant throughout the experiments. From this it might be argued that the nitrogen retention observed in such experiments is without direct influence on the endogenous protein metabolism.

b. The distribution of sulfur in the urine: In ordinary procedures for the analysis of the urine, the sulfur is usually divided into three fractions, the inorganic sulfate sulfur, ethereal or conjugated sulfate sulfur, and unoxidized or the so-called "neutral" sulfur. Of these three fractions, the inorganic sulfate sulfur forms by far the greatest part. For example, Folin (48) in a series of analyses of urines from normal individuals on relatively high protein diets (average daily nitrogen excretion in the

urine, 16.0 grams) recorded the following average figures for the distribution of sulfur in percentages of total sulfur: inorganic sulfate sulfur, 87.8 per cent; ethereal sulfate sulfur, 6.8 per cent; neutral sulfur 5.1 per cent.

Of these, the inorganic sulfate sulfur was most variable and as shown by Folin (49) increased or decreased in absolute amount with the increase or decrease in the urea excretion. It is generally accepted that any factor which influences protein metabolism and the elimination of urinary urea will also affect the excretion of inorganic sulfates since both of these urinary constituents have a common origin in the protein molecule, the one from the nitrogen, the other from the sulfur. As far as is known, the character of the protein metabolism is not sufficiently altered by work as to change this relationship between urinary urea and inorganic sulfates (147). Scott and Hastings (145), however, considered that the elimination of sulfur might be increased in severe muscular work because of the function of the sulfur complex of the protein molecule in respiratory processes in the cell. Their results indicated "a tendency for the proportion of total sulfur (which is eliminated as sulfates) to increase during a day of exercise; and this tendency is more marked; the more severe the exercise." They also noted an undoubted increase during the day in the output of sulfate sulfur per gram of nitrogen in men subjected to labor and no increase in men at rest in bed. Their data were obtained from hospital patients and industrial workers of various types, whose diets were not controlled. The samples of urine analyzed were random samples taken at the same hour daily. In view of the recent work on the relation of the sulfhydryl group to oxidation, a repetition of these experiments with controlled diets and analysis of complete 24-hour samples of urine is desirable.

The conjugated or ethereal sulfates present in the urine have usually been considered as products formed in the detoxication of the toxic phenol and indol derivatives of bacterial origin in the intestine. A small amount (5 mgm. per day in man) of sulfur may be eliminated as conjugated sulfates in the form of chondroitic acid (130). The conjugation of the bacterial products is thought to occur primarily in the liver (44) and the sulfuric acid used, to be withdrawn from the inorganic sulfate fraction. Konschegg (88), however, considers that a part of the ethereal sulfates may be formed in the intestines. The reported occurrence of ethereal sulfates in bile does not hold for all species (20).

The source of the sulfuric acid used in conjugation is not definitely

known, despite much experimental work. A comparative study of the excretion of ethereal sulfates after the administration of phenol alone and of phenol with various sulfur compounds indicates that the oral administration of colloidal sulfur may cause a significant rise in ethereal sulfates (87), (138). Despite the fact that the sulfur of thiourea is not oxidized by the animal organism, its administration either per os or subcutaneously is reported to increase the ethereal sulfates of the urine (87), (109) although negative results with thiourea are also reported (138). The most marked influence on ethereal sulfate elimination was observed, as was to be anticipated, after the administration of cystine with phenol (131), (138). The fact that no increase in conjugation was observed after administration of sodium sulfate[2] with the phenol (131) suggests that conjugation may occur previous to the complete oxidation of the sulfur of the cystine molecule. The administration of sodium sulfite with phenol increased the extent of conjugation almost as much as did administration of cystine (131).

Although it must be regarded as definitely proven that a part at least of the ethereal sulfates owe their origin to products of bacterial activity, the possibility of an endogenous origin of a part of this sulfur cannot be excluded. Folin (49) observed that the ethereal sulfate fraction became more prominent when the food contained little or no protein and concluded that the ethereal sulfates could be due only in part to intestinal putrefaction. However, as pointed out by Hopkins (76), we have no evidence that a decrease in the activity of the intestinal flora is necessarily associated with a restriction of the protein content of the diet. Konschegg (88) suggested that in the presence of an excess of sulfates in the system, the production of phenol of endogenous origin might occur, just as the presence of benzoic acid results in glycine for hippuric acid synthesis. The possibility of an endogenous origin of conjugated sulfates has also been suggested by others (87), (121), (131). It is of interest to note that the administration of cystine with brombenzene decreases the output of ethereal sulfates as compared with that when brombenzene alone is fed (121), (131). Cystine with phenol, on the other hand, gives a greater output of ethereal sulfates than when phenol alone is fed.

[2] Hele (Journ. Physiol., 1923, lviii, p. xlvi) has recently shown that the following administration of the relatively non-toxic guaiacol in large doses with inorganic sulfate, from 60 to 80 per cent of the administered sulfate may appear in the urine as conjugated sulfate. Full details of this investigation have not yet been published.

The recent discovery by Neuberg and his associates (123), (124) of a sulfatase in Takadiastase, which can hydrolyze ethereal sulfates, suggests the possibility of a more widespread distribution of this enzyme and its possible rôle in the body. The fact that the salts of phenylsulfuric acid are eliminated almost quantitatively as such after administration to animals (30), (131) would show the absence of such a reaction in the organism of the higher animals.

The unoxidized or neutral sulfur fraction of the urine is more constant in amount than either of the other two sulfur fractions. Folin (49) in 1905 called attention to this fact and suggested that the neutral sulfur was associated with some process of endogenous metabolism and was as truly characteristic of endogenous metabolism as creatinine. Among the substances whose sulfur has been considered to be determined in this fraction, are cystine, taurine or derivatives, bile acids, thiosulfates, thiocyanates, urochrome and a group of more complex organic substances of unknown composition, the various oxyproteic acids and related compounds.

That taurine and bile acids contribute materially to the neutral sulfur of the urine has been argued from the fact that taurine, as previously discussed, is eliminated unchanged in the urine of man or the dog, and from the experiments of Kunkel (90), who observed a marked drop in the elimination of neutral sulfur after the establishment of a biliary fistula in a dog, a decrease which corresponded almost exactly to the amount of sulfur secreted in the bile and withdrawn from its normal path of absorption from the intestine by the fistula. Shaffer (146) studied the metabolism of a woman with a permanent biliary fistula. The amount of neutral sulfur eliminated in the urine was as great as that excreted by normal individuals. No increase in urinary neutral sulfur was observed when considerable amounts of the patient's own bile were injected into the intestine through the fistula or after the oral ingestion of 20 grams of dried ox bile. These studies, which failed to show any relationship between the sulfur of the bile and the neutral sulfur of the urine, receive further confirmation from the fact that the ingestion of taurocholic acid by the dog (141) did not increase the neutral sulfur excretion, and that appreciable quantities of bile acids could not be detected in the urine even in icterus (143).

No satisfactory methods for the determination of cystine in urine have been available until recently, so that few figures are available for the cystine content of normal urines. Recent studies (104) in which a new colorimetric method developed by Looney (103) in the laboratory

of Folin was employed for cystine estimation, show in normal urines, a cystine content of from 0.0 to 12.0 mgm. per 100 cc. of urine, and a 12-hour excretion of cystine by the fasting individual of about 30 mgm. If these findings are typical, cystine is hardly an important substance among those which contribute normally to the neutral sulfur of the urine.

c. Cystinuria: The cystinuric individual, on the other hand, may excrete very significant quantities of cystine in the urine. Little advance has been made in our knowledge of this baffling "error of metabolism" since the classical paper by Garrod (55) in 1908. Certain facts which are generally agreed upon may, however, be stated briefly.

1. The cystine excreted is in part of endogenous origin, since ingested cystine is readily oxidized by the cystinuric to sulfates (14), (173), and does not appear in the urine as such (104). In the case reported by Thiele (162), cystine isolated from the patient's own urine was fed to the patient and was completely oxidized. The most notable exception to the above is the case of Loewy and Neuberg (105), in which 5.0 grams of cystine (from hair) were quantitatively eliminated as such in the urine after oral administration. After feeding 3.52 grams of cystine prepared from a cystine calculus, no extra cystine was found in the urine. This difference in the case of oxidation of cystine derived from different sources can hardly be explained except on the basis of the older idea that protein cystine and stone cystine are distinct but isomeric substances, a theory which is not borne out by more recent work (4). Hoffman and Gortner (75), however, have succeeded in preparing a "needle" cystine, isomeric with "plate" cystine, from the latter by prolonged boiling with acid. Further evidence for endogenous origin of cystine in cystinuria is to be found in the continued excretion of cystine in starvation or on a protein-free diet (14).

2. The absolute amount of cystine excreted appears to depend to some extent upon the amount of absorbed protein (14), (170), (173). This has been considered to indicate that the cystine complex of the protein molecule is absorbed as a peptide or in some form other than as cystine since cystine, when free, is oxidized completely.

3. In some individuals, a disturbance of the catabolism of amino-acids other than cystine occurs, since the diamines, putrescine and cadaverine, appear in the urine. In the case of Loewy and Neuberg (105), although no diamines were found in the urine under ordinary conditions of diet, after the administration of arginine and lysine, the

corresponding diamines were excreted by the kidneys. Tyrosine and aspartic acid, after oral ingestion, were also excreted, largely unchanged in the urine. In most other cases, the catabolism of amino-acids other than cystine was normal (14), (170), (173). The amino-acid nitrogen content of the urine was not increased above the normal (104).

4. Not all the sulfur compounds liberated in the processes of endogenous metabolism escape oxidation, since a very considerable amount of sulfate appears in the urine.

The failure of the organism to oxidize one specific amino-acid in cystinuria would support the theory that the processes of deamination and oxidation are not the same for all the constituent amino-acids of the protein molecule. No entirely acceptable theory has yet been advanced by which the failure of the oxidation of cystine can be explained. The recent suggestion of Abderhalden (7) that the cystinuric individual may be lacking in the ability to convert cystine to cysteine as the initial step in the utilization of cystine should be subjected to experimental study.

6. THE FUNCTION OF CYSTINE IN THE ANIMAL ORGANISM. The current theory of metabolism holds that the "wear and tear" quota of endogenous protein metabolism represents the breakdown of body tissue to supply certain amino-acids essential for vital activity, when these are not supplied to the organism in the diet. Thus tyrosine is supposed to be required as the precursor of adrenalin, tryptophane for the synthesis of thyroxin. How does cystine, an amino-acid required for growth in young animals and for nitrogenous equilibrium in adults, function in cell activity? Current teaching assigns to cystine and its reduction product, cysteine, an important rôle in the regulation of animal oxidations and reductions. The rôle of the sulfur-containing fraction of the protein molecule and of sulfhydryl groups in general was emphasized by Heffter (72) and later by Thunberg (164) to whose excellent review of the subject reference may be made for the earlier literature. The theory of the importance of the mercapto group of cysteine in physiological oxidations and reduction was placed upon a firm foundation by Hopkins (77) by the actual isolation of a dipeptide, glutathione,containing cysteine and glutamic acid, from yeast, muscle and liver tissue. This was shown to function as a coenzyme in oxidative and reductive reactions. For an account of the discovery and properties of glutathione, the reader is referred to the recent summary by Dakin (34) in these REVIEWS. Further work from the laboratory of Hopkins (40), (78) has confirmed and extended the earlier work.

The isolation of glutathione is tedious and difficult, but fortunately a delicate reaction, which is almost specific for the presence of mercapto groups, is available in the nitroprusside test. Studies in which this reaction has been applied as a presumptive test for the presence of glutathione have added materially to the accumulated evidence concerning the relation of glutathione to oxidation. Hopkins (77) demonstrated that the hen's egg gave no reaction with the nitroprusside, but that after 30 hours' incubation, when the development of the embryo had begun and oxidative processes presumably were increased, the embryo gave a strongly positive nitroprusside reaction. Still more striking are the experiments of Shearer (148) on the effects of fertilization on the eggs of echinoderms (Echinus miliaris). The ripe sperm and eggs contained appreciable amounts of glutathione as evidenced by the nitroprusside test. One minute after fertilization, before the sperm had actually penetrated the ovum, the oxygen consumption had increased to 8000 per cent of that of the unfertilized egg and the nitroprusside reaction was very intense. In immature eggs and sperm, the reaction was negative. The substance which gave the nitroprusside test could be washed completely out of the cell by isotonic solutions containing acetic acid.

The experiments of Abderhalden (7) show that part of the substance in tissues which is responsible for the nitroprusside reaction is dialyzable and not bound with the protein. A quantitative estimation of the cysteine or substance reacting with nitroprusside in horse muscle (8) showed the presence of about 100 mgm. (calculated as cysteine) per kilo of muscle. Hopkins (77) isolated 100 to 150 mgm. of glutathione per kilo, equivalent to 45 to 67 mgm. of cysteine. Presumably free cysteine or cysteine in other forms than glutathione may be present in tissues although no claim for quantitative isolation of glutathione is made by Hopkins (77). Peptides containing cystine also react as does cystine in their capacity to act as coenzymes in oxidation and reduction (11). Abderhalden (10) has also obtained evidence that the presence of cystine or cysteine may markedly influence the course of the action of tissue extracts upon aldehydes in the Cannizzaro reaction (aldehydemutase of Parnas and Neuberg). Although the exact details of the mechanism of the reaction are not agreed upon (11), (77), (78), (112), all investigators agree in assigning to the equilibrium, cystine $\rightleftarrows$ cysteine, an important rôle in oxidations and reductions of the body.

The high content of the epithelial protective tissue in sulfur and cystine, a content greater than that of any other tissue or pure protein,

suggests that the cystine in this tissue may have a specific function. Paramecia (68) were subjected to ultra-violet light which had previously passed through solutions of cystine in sodium hydroxide. The time of exposure required to produce fatal results was 1200 seconds if the ultra-violet rays first passed through the cystine solution as compared with 150 seconds in the controls. From this it is argued that susceptibility of protoplasm to ultraviolet light is conditioned by the selective absorption of the toxic rays by cystine (and certain aromatic amino-acids). Ward (167) in his studies of the absorption spectra of amino-acids, found that cystine was the only amino-acid which had any marked absorption in the region of solar ultra-violet light. It would appear that the presence of relatively high concentrations of cystine in wool and hair is of physiological importance in the protection of the organism against the harmful effects of prolonged exposure to sunlight. Ward has called attention to the fact that the curious custom of the Arabs in wearing heavy woolen clothing may have a strong justification in the protective effect of the cystine in the wool.

7. SULFUR METABOLISM IN RELATION TO DISEASE. There is little evidence of specific pathological changes in the sulfur metabolism in disease. Inasmuch as the greater part of the sulfur of the urine is present as inorganic sulfates and is related to the urea nitrogen in the processes of the catabolism of protein, any change in protein metabolism in disease must of necessity involve a change in the sulfate sulfur formed and eliminated. This, however, must be regarded as a consequence of general alteration in protein metabolism rather than a specific change in sulfur metabolism. The claims of a specific increase in the neutral sulfur fraction of the urine in carcinomatous individuals (137) have not been satisfactorily established (63), (157).

Abderhalden and Wertheimer (9) have shown that, in pigeons fed on a diet of polished rice exclusively, there occurs a failure to utilize the oxygen of the blood normally, accompanied by a lowered body temperature and a depression of the gaseous metabolism. In the tissues of these animals, the nitroprusside reaction was weak or negative, while the tissues of starving pigeons gave strongly positive reactions. A study of the ratio of the carbon, nitrogen and sulfur in the tissues showed that the absence of the nitroprusside reaction was not to be attributed to lack of sulfur (and cystine). Aqueous extracts of the tissues of normal birds were able to reduce added cystine to cysteine (as shown by the nitroprusside reaction). The tissue extracts of the rice-fed pigeons could not effect the reduction of cystine. From these

experiments, it would seem that one factor, at least, in the lowered metabolic processes lay in the inability to utilize cystine for the production of cysteine, which has an important rôle in oxidative processes of the tissues. Abderhalden suggests that normally, the reaction R.S−S.R ⇄ R.SH+HS.R is a balanced one; that in "alimentary dystrophy" (the term applied to the condition of malnutrition resulting from lack of specific substances in the diet, vitamines) in the rice-fed birds, the reaction proceeds in one direction only, R.SH + HS. R→R.S − S.R., reversal of the reaction is prevented and mercapto groups necessary for the oxidation are not available; and that in cyanide poisoning, the reaction can proceed only in the opposite direction, R.S.−S.R→R.SH + HS.R, and that no utilization of the mercapto groups present for oxidation is possible, since cyanides prevent conversion of the mercapto group to disulfides. The ultimate result in cyanide poisoning and in alimentary dystrophy is the same, failure of oxidation, but, as stated, the mechanism by which oxidation is prevented is different.

It is of interest to note that in rats on synthetic diets from which cystine was absent (5), nutritive failure was accompanied by an abnormally weak nitroprusside reaction in the tissues. Mention should also be made of the case of cystinuria reported by Abderhalden (1) which terminated fatally, due to general failure of nutrition.

Among the tissues of the body which give a strongly positive nitroprusside reaction, is the lens of the eye (15). Goldschmidt (62) has studied the cysteine content of the lens of cattle at various ages, as determined by the ability of the tissue to form hydrogen sulfide from elementary sulfur (72) according to the general reaction,

$$\begin{matrix} R-SH \\ R-SH \end{matrix} + S = \begin{matrix} R-S \\ | \\ R-S \end{matrix} + H_2S$$

The cysteine content of the lens, as determined by this method, varied little in calves between the ages of 8 days and one year, but there was a diminution of the cysteine content of the lens in adult life (1.3386 per cent cysteine in the lens of calves, 0.5820 per cent in the lens of cattle 8 to 17 years of age). No differences could be observed in the total sulfur content of the lens at different ages. In cataract of the lens, however, the nitroprusside reaction was absent (7) and the lens was unable to reduce elementary sulfur to hydrogen sulfide (62). If the lens was first treated with sodium sulfite (which reduces cystine to

cysteine) the cataract tissue behaved as a normal lens, thus demonstrating that the failure of hydrogen sulfide formation was due, not to a cystine deficiency but to the inability to form cysteine. Goldschmidt considers that the amount of cysteine present may determine the oxidative processes of the lens and play an important rôle in the pathology of cataract.

The sulfur content of various melanins is well known. Brahm (25) has recently found 3.42 per cent of sulfur in melanin from a tumor and suggests that the pigment may be a phenyl derivative of cysteine, 3, 4-dihydroxyphenylcysteine.

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THE NUTRITIVE VALUE OF PROTEINS

H. H. MITCHELL

University of Illinois College of Agriculture, Urbana

The complete determination of the nutritive value of any food material involves a determination of its content of total nutrients, and of the losses these nutrients sustain in the course of their assimilation by the animal body. For example, the values of stock feeds as sources of energy to the animal are completely expressed by their so-called "net energy" values, since the latter are obtained by deducting from the gross energy of the feed all of the losses incurred in digestion and metabolism. Such measures of nutritive value are obviously subject to considerable variation for different animals under like conditions, and may be profoundly affected by changed conditions. Again referring to the energy value of farm feeds, since these have been studied more systematically than the energy value of human foods, the net energy value of a feed is closely dependent upon the species of animal, the purpose for which the energy is being used, and probably also upon the age of the animal and the level of food intake. Unfortunately the problem of equating food intake with bodily requirements can never be reduced to a simple and exact mathematical operation.

The wastage of food protein in digestion. In the utilization of food protein by animals, just as in the utilization of food energy, a certain wastage seems to be almost inevitable. The first wastage is due to incomplete digestibility. The task of measuring the extent of this waste is complicated first, by the fact that the nitrogen of the feces is the only constituent that can be related to the protein consumed, though all of the food nitrogen may not be contained in its proteins; and second, by the fact that a highly variable fraction of the nitrogen contained in the feces is of body origin, or is contained in bacterial residues. Evidently the more indigestible the food protein is, the smaller the proportion of body nitrogen contained in the feces, while the smaller the intake of protein, the greater the proportion of body nitrogen in the feces.

The ordinary method of expressing the digestibility of food protein is as the percentage of the nitrogen consumed that does not appear in

the corresponding fecal excretion. While the coefficient thus obtained ignores entirely the contribution of the body to the nitrogen of the feces, the so-called "metabolic nitrogen," it undoubtedly possesses a great deal of practical value by virtue of the fact that this "metabolic nitrogen" is after all in much the same category as the actual undigested food nitrogen in that it represents to a large extent wastage of nitrogen consequent upon the digestion of the food. Calculated on this basis there are large differences in the digestibility of the proteins of different food materials. The proteins of meat and milk seem to be very completely digested, when not dried or overcooked; the proteins of egg white, on the other hand, are only about 58 per cent digestible under the most favorable conditions (121), and when consumed in the raw, unbeaten condition they are less digestible (4). The proteins of cereals and vegetables occupy an intermediate position with respect to the completeness of digestion and exhibit a good deal of variation (123). The proteins of the legumes are of relatively low digestibility, ranging from 70 to 80 per cent, while the proteins (nitrogen) of many fruits, such as the apple, prune and strawberry, are extremely indigestible.

However, these differences in the apparent digestibility of food proteins do not in the main depend upon chemical differences existing among them, but rather upon the presence in the food of indigestible carbohydrates, such as celluloses, hemicelluloses and pentosans. Mendel and Fine (75), (76), (77), (78), (79), (80) have shown that these differences largely disappear when the vegetable proteins are fed in a more nearly pure condition. They found that the proteins of wheat were as well utilized as the proteins of meat, and that the proteins of barley and of corn were also probably as well digested. With regard to the proteins of soy beans, navy beans (cooked) and peas, they concluded that the presence of indigestible non-nitrogenous materials cannot entirely account for their low coefficients of digestion. These proteins appeared to be less readily and completely disintegrated by the digestive processes than the cereal proteins. This resistance to enzyme hydrolysis was even more pronounced with cottonseed proteins.

An interesting light has been thrown upon the digestibility of the proteins of several leguminous seeds by recent investigations of Jones, Waterman and Johns. In these studies on the digestibility of proteins *in vitro* it has been shown (149), (150) that the rate of digestion of phaseolin, the most important globulin in the navy bean, and of the proteins of the velvet bean, is much improved by cooking. The value

of these proteins (37), (14) and of the proteins of the lima bean (13) and of the cow pea in large part depended upon whether or not the proteins were cooked. No explanation of this effect of cooking has been given, since it is not predictable from any of the known properties of proteins. The same investigators have found (45) that arachin, the main globulin of the peanut, occupies an anomalous position among the proteins thus far investigated by reason of its great resistance to the action of proteolytic enzymes, a resistance that is not diminished by previous cooking of the protein. It appears that in the course of its hydrolysis, a fragment is formed which is digested with great difficulty, possibly due to the stereochemical arrangement of its amino acid components.

The relative indigestibility of the proteins of the cottonseed, reported by Mendel and Fine (79), was found by Jones and Waterman (46) to be due to the presence of gossypol, which exerts a marked inhibitive effect upon proteolysis. In its absence cottonseed globulin appears to be as well digested as casein.

The significance of determinations of the digestibility of the nutrients in different foods is limited by the fact that, when two or more foods are consumed together in a mixed diet, there is frequently a marked effect of one food on the digestibility of another, particularly if the different foods differ greatly in texture and composition. Frequent illustrations of this fact may be cited from the literature, particularly from the numerous digestion experiments on farm animals, where the roughage fed generally exerts a marked effect on the digestibility of a concentrate, and *vice versa*. A striking effect of milk on the digestibility of the proteins of corn is shown in a recent experiment by Hart and Steenbock (26) on swine. As the percentage of milk protein was increased from 3.2 per cent to 28.5 per cent of the total protein in the ration, the coefficient of digestion of the protein mixture increased from 55 to 83, and of the corn proteins from 49 to 77, assuming complete digestibility of the milk proteins. Many illustrations of "associative digestibility" have been reported and discussed by Rubner (124). Obviously in view of this situation, the computation of the digestibility of the nutrients of one food from the digestibility of the nutrients of a mixed diet and of the other foods with which it is consumed, cannot possess any great degree of accuracy and frequently leads to grossly inaccurate if not impossible results.

The wastage of food protein in metabolism. While the wastage of food protein in digestion seems to be largely unrelated to its chemical structure, the wastage of food protein in metabolism in covering the protein requirements of the body is generally ascribed entirely to its chemical structure. The value of the digestible protein of a food in meeting the protein requirements of the body, allowance being made for this wastage of protein in metabolism, has been called the "biological value." Like the coefficient of digestibility, it is expressed as a percentage.

That different proteins possess different biological values may be accepted today as one of the fundamental facts of the science of nutrition. The fact of such nutritive differences is entirely consistent with the accumulating evidence of differences in the amino-acid make-up of proteins. In the case of many individual proteins, especially those totally lacking in certain amino acids that cannot be synthesized by mammals, or extremely deficient in such acids, the differences in biological value may be accounted for in large part by known differences in chemical constitution. Hence the opinion is widely held that the biological values of proteins are completely determined by the proportions of the different essential amino acids resulting from their complete hydrolysis.

However, this conception has not met with complete success in explaining the demonstrable differences in biological value existing among some proteins. For example, the low biological value of arachin (45), (133), the main globulin of the peanut, has not been so explained. On the other hand, the differences in amino-acid make-up existing among animal proteins and the proteins of the cereal seeds would lead one to infer a much greater difference in biological value than appears to exist. If proline is a limiting factor in the biological value of edestin (140), the relatively high content of this amino acid in edestin is anomalous. Osborne has pointed out (95, p. 99) that the relative nutritive values of phaseolin and of casein are not readily explained by reference to their chemical composition.

If it were perfectly obvious that the biological value of a protein must depend solely upon its constituent amino acids and the proportions in which they occur in it, such discrepancies as those indicated would have no significance except as an expression of the incomplete information available as to the chemistry of proteins and protein mixtures. However, such is not the case. While it cannot be doubted

that the biological value of a protein is limited very definitely by its amino-acid make-up it is also true that it *may* be modified by other factors also. As the author has stated elsewhere (87):

> It is generally believed . . . . . that the chemical constitution of a protein determines entirely its biological value. This belief tacitly assumes that the economy with which the cells utilize the amino acids supplied to them is unaffected by the abundance of the supply, particularly by the rate at which the amino acids are transported to the cells from the intestinal tract. It also assumes that the undigested fraction of a dietary protein is representative in its amino-acid make-up of the protein as consumed, so that factors influencing the digestibility of the protein would have no effect on the proportions of the different amino acids absorbed into the blood. It must also assume that no great differences in the rate at which different amino acids are liberated from a protein during digestion occur, or that if such differences do occur, the storage capacity of the tissues for absorbed amino acids is sufficiently great to insure the presence there throughout the digestive period of optimum proportions for synthetic purposes of the several amino acids. It also assumes that until the demands of the body for protein are covered by the protein intake, amino acids are not [preferably, *need not be*] oxidized in the body except in so far as they are unavailable for synthetic purposes, and hence that factors affecting the intensity of the oxidation processes can have no effect on the utilization of amino acids for structural purposes. These assumptions are either extremely improbable or highly debatable.

If one function of the liver, as Van Slyke believes (148), is to deaminize amino acids coming to it in the portal blood, even before the immediate protein requirements are met, this may constitute another biological factor influencing the biological value of proteins.

The net protein value of a food. It is reasonable to regard the biological value of a protein as quite analogous to the coefficient of digestion. By the combined use of average percentages of total protein in foods, average digestion coefficients, measuring the wastage of protein in digestion, and average biological values, measuring the wastage of protein in metabolism, it is possible to compute what has been termed (83), (84), (89) the "net protein" content of a food. For example, if a cut of beef contains 20 per cent of protein of which 95 per cent is digestible, it would contain 19 per cent of digestible protein. Now if only 80 per cent of the digestible protein (N $\times$ 6.25) could be used to cover the protein requirement of the body at ordinary levels of protein intake, then the meat could be said to contain $19 \times 0.80 = 15.2$ per cent of net protein. Navy beans with 22 per cent of protein, of which 80 per cent is digestible, and of the latter only 38 per cent is available for body purposes, would contain 6.69 per cent of net protein. While

it may be found that the net protein value of foods would be so greatly affected by the conditions of feeding and the purposes for which the protein would be used in the body as to deprive it of any general significance in practical dietetics, it is important to emphasize that a low biological value of its protein does not condemn a food as a source of this nutrient since a high content of protein of good digestibility may quite offset the great wastage in tissue synthesis. This wastage, of course, refers only to the process of tissue maintenance and growth. No wastage of energy is implied.

The nitrogen content of a food as a basis for the computation of its protein value. The determination of the nutritive value of isolated proteins and of the mixed proteins of food materials has been pursued along two general lines: one involving a comparison of the protein content of a ration, or of the actual protein intake, with the changes in weight of the experimental animal; the other involving a more thorough analysis in the direction of a determination of the digestibility of the protein and of the biological effect of the absorbed fraction. This latter analysis can only be carried out on one constituent of protein, namely, its nitrogen. Unfortunately not all proteins have the same nitrogen content and not all of the nitrogen of a food is contained in its protein.

In meeting this situation it may be argued, on the one hand, that it seems as fair to compare proteins on the basis of their nitrogen content as on their dry weight. In planning synthetic rations for the determination of the relative nutritive values of a series of isolated proteins, it would seem to be as well to incorporate equal percentages of protein nitrogen as equal percentages of protein. The conception of the protein value of a food as being well represented biologically by its nitrogen content multiplied by a factor (6.25) such that, if the nitrogen is completely utilized in digestion and metabolism, the product will give the equivalent of body protein, is not without justification, even though it was originally adopted merely as a chemical expedient.

On the other hand, the non-protein nitrogenous substances cannot be neglected in assessing the nutritive value of a food as a source of protein, since these substances, consisting so largely of amino acids and amino-acid derivatives (16), may supplement the true proteins or may even possess a value of their own in satisfying the protein requirements of animals. Certainly the protein value of a food like the potato, in which 40 per cent or more of the nitrogen is in non-protein forms, is much more adequately represented by its content of crude

protein than by its content of true protein. For these reasons, the "crude protein" (N × 6.25) content may have greater significance in the biological evaluation of a food than its true protein content; similarly the determination of the protein value of a food by tracing the disposition of its nitrogen in digestion and metabolism may be adequately defended.

In reviewing the literature that has accumulated in the last decade or so relative to the nutritive value of proteins and protein mixtures, one is struck with the number of contradictory conclusions. On the one hand are found general statements that the proteins of milk and of meat are two to three times as valuable to the animal organism as the proteins of cereals, while on the other hand no great disparity is said to exist between them. In fact, one investigator considers that the proteins of wheat are superior to those of milk, and that meat proteins are to be placed in the same category as the cereal proteins. There may be found evidence that milk proteins are completely utilized in covering the so-called maintenance requirement for protein, and still other evidence that they are utilized for this purpose only to the extent of 50 per cent. A mere summary of such evidence and such conclusions can serve no useful purpose. A critical study of the evidence indicates that much of the confusion is due to the employment of several different methods of investigation, some of which are not particularly effective in that the results obtained cannot be definitely interpreted. Some of the confusion is due to a failure to consider the different functions that protein may serve in the body.

METHODS OF INVESTIGATION. *The biological method for the analysis of a foodstuff.* A method that has been used most extensively in recent years to determine the nutritive value of the proteins of different foods, as well as their value as sources of the other nutrients, is the so-called "biological method of analysis." This method is used with rats as subjects and is based upon a working hypothesis concerning the necessary components of a complete diet formulated by McCollum and Davis in 1915 (57). According to this hypothesis, a complete diet for the rat must contain a sufficient amount of protein of good quality, sufficient energy, adequate amounts of a complete salt mixture, and adequate amounts of two unidentified factors, vitamins A and B. In judging the protein value of a food, the ration used is designed to contain 9 per cent of protein, since even with the best quality of protein this level of intake will just support maximum growth. The

ration is made to contain the factors other than protein in adequate amounts, and the results obtained with respect to growth, fertility, success with the rearing of young, longevity, the preservation of youthful characters and the stability of the nervous system, are interpreted with reference to the quality of the protein intake. The investigations have sometimes been carried through the second, third or fourth generation, the general well-being of which is another contributing factor to the final decision of the biological adequacy of the protein consumed.

In his later work McCollum has modified his original working hypothesis in that, instead of using a complex mineral mixture in his rations to insure an adequacy of such nutrients, he adds only calcium carbonate and sodium chloride, unless the food under investigation constitutes such a small percentage of the ration that a complete mixture is obviously essential. His justification for this simplified procedure, as far as the author is aware, rests entirely (55, p. 29) upon some experiments on the mineral inadequacies of a mixture of maize and navy beans (58). The extension of the conclusions deduced from these experiments to the cereal and legume seeds in general, and later to animal tissues, rests on the similarity in reported inorganic analyses of these different food materials.

The large amount of work that has been done with this method for the biological analysis of a food (or some modification of it) by McCollum and his associates, and by other investigators as well, warrants a somewhat detailed consideration of it. The great merit of the method, in the opinion of McCollum, is its greater refinement. It is claimed that differences in the biological values of different proteins may be demonstrated with this method that observations on growth alone will fail to reveal (67, p. 237).

However, many serious objections may be raised against the effectiveness of the method.

The method is based upon a theory of nutrition that is known to be inadequate. Since the formulation of this theory it has been shown that there are two fat-soluble vitamins (132) and that very probably the unidentified factor known as vitamin B also is dual in nature. It is premature even now to attempt to specify how many unidentified dietary factors are required for the successful nutrition of both sexes throughout their normal span of life. In particular, there is some evidence of the specific need of a vitamin for reproduction (12), (141). If this be true, the reproduction records of rats on rations adequate in all known dietary factors with the possible exception of protein, cannot

be referred with any degree of certainty to the quality of the dietary protein, since they may also be dependent upon the presence in the food of adequate amounts of an unknown dietary factor essential for this specific animal function. The function of lactation may also, for all that is known to the contrary, be dependent upon unknown dietary factors, while so little is known of the relation of nutrition to the preservation of youthful characters, to longevity, and in particular to the maternal instincts that any theory concerning this matter is pure dogma. It should be remembered that while the nutritive requirements of the rat for growth seem to be fairly well standardized, such cannot be said to be true for the entire life cycle of this animal.

While the matter of mineral requirements is ordinarily assumed to be fully met by the several mineral mixtures commonly used in the preparation of experimental synthetic rations, the possibility is still not excluded that small amounts of certain inorganic ions occurring in mere traces in animal tissues (49) may not also be indispensable, and that ultimate failures in nutrition on rations adequate with respect to all known dietary factors except protein, may not at times be due to such inorganic deficiencies rather than to the quality of the dietary protein.

The assumption made in all of McCollum's recent work on the nutritive and supplementary values of proteins that seeds and animal tissues may be adequately supplemented with respect to their content of inorganic elements by additions of sodium chloride and calcium carbonate only, is particularly open to criticism, since the experiments cited in support of this assumption (58) were restricted to one particular combination of seeds and were concerned in the main only with growth. A ration of maize and beans supplemented with butterfat, sodium chloride and calcium carbonate (58, chart 17) permitted normal growth and reproduction, but the rearing of the young was unsatisfactory (36 per cent). Whether this unfavorable result was due to inadequate protein, minerals, or some unknown factor cannot be decided from the evidence offered.

The question of the function of roughage in the diet is still unsolved apparently (90), so that it cannot be decided that this factor also might not be operating in the ultimate results secured with rations containing animal tissues unsupplemented in this respect.

Until the nutritive requirements of the rat for the entire span of life and for all physiological functions are at least as well known as the requirements for maximum growth, this method for the biological

analysis of a food as a source of protein cannot be used with any great assurance of success unless proper control experiments are run at the same time. If the addition of an adequate amount of a protein of good quality to a ration containing all known dietary factors in adequate amounts with the possible exception of protein, which is provided by the food under examination, gives a ration capable of supporting optimum nutrition, any inadequate nutrition evident from experiments on the original ration can logically be attributed to the inadequacy of its protein, *provided the food intakes in the two experiments are comparable.*

Thus controlled, the method will permit a more definite interpretation of the results secured. However, the comparative value of such experimental results would still be debatable, because the protein requirements for growth, reproduction and lactation are in all probability qualitatively as well as quantitatively different. Quite in accordance with this view may be cited some experiments by McCollum, Simmonds and Parsons (61) in which combinations of certain food proteins did not give any better growth than could be obtained with either food protein alone, but did show a distinct improvement in the reproduction records, and other experiments by the same investigators (64) showing that high fertility was generally, though not always, associated with good to excellent growth, while high mortality of the young is frequently associated with excellent growth and high fertility.

The method, therefore, attempts to gauge the nutritive value of proteins by different standards, and hence gives results that are not comparable. Obviously the deficiency of a protein in covering the requirements of growth can hardly be compared with the deficiency of another protein in covering the requirements of reproduction or lactation, if these requirements are qualitatively different. If wheat protein, for example, is particularly effective in securing optimum fertility of females or in making possible the successful rearing of the young, this property may be totally unrelated to its value in growth, so that proteins which are inferior to it in the former respect, as milk proteins are said to be, may be distinctly superior to it in the latter respect. The protein requirements for growth, maintenance, reproduction and lactation are each distinct problems and should not be confused or merged with one another.

A nutrition experiment concerned with the relative suitability of different foods in covering the nutrient requirements of the body should be concerned with a comparison of the intake of the food, or of some

nutrient contained in it, if this can be determined by chemical analysis, with some physical or physiological measurement of the experimental animal, such as body weight or the rate of retention of nitrogen. Food intake records are thus a part of the experimental evidence. There are some nutrition experiments in which food intake records are of minor importance, but whenever *quantitative* information is desired, obviously an accurate knowledge of the amount of food consumed is essential. In many cases a controlled food intake is highly desirable in order to permit an intelligent interpretation of the results obtained. The problem of the relative nutritive value of different proteins and protein mixtures involves the measurement of the value of each protein and protein mixture in some common unit, and hence is a quantitative problem requiring quantitative methods for its solution. A failure to appreciate this fact is responsible for a large number of experimental reports in protein nutrition, the conclusions of which cannot be intelligently reviewed.

The biological method of analysis of the protein value of foods is ordinarily conducted in such a way that the animals can consume as much of the food as they desire, and the results obtained in the way of growth curves, reproduction records, etc., are interpreted entirely without reference to the amount of food consumed, even though this may have been accurately determined. In defending this procedure against the very criticism that is here made, McCollum (55, p. 107) cites the experience of Osborne and Mendel (100), leading them to believe that the food intake of the rat is determined in large measure by its energy requirement and the calorific value of the food. Hence, it is argued that if the rations used in the determination of the nutritive value of different food proteins possess approximately the same energy value, it may be assumed that the food intakes are entirely comparable and may therefore be disregarded. However, this opinion of Osborne and Mendel, reported in 1915, was based upon experiments with synthetic food mixtures possessing little if any flavor that would enhance or diminish the appetite, and, in fact, was later modified (109) as the result of experiments indicating the operation of other factors in determining the amount of food consumed by rats.

Such experiences, however, cannot furnish reliable information relative to the amounts of food that will be consumed by rats subsisting on rations containing natural foods to an extent that will impart a distinctive flavor. As a matter of fact, there are numerous instances cited in the literature of nutrition experiments on rats, of

rations being refused because of distasteful flavors imparted to them by natural foods (see particularly 53, p. 152), and of the favorable effect of cooking on the amount of a given ration that will be consumed, apparently because of a modification of flavor.[1] Certainly with such rations the burden of proof that the energy value solely determines the amount that will be consumed must be assumed by the investigator himself. The question cannot be dismissed with a gesture.

For the reasons enumerated above, the conclusion appears justified that, unless the method of the biological analysis of foods proposed by McCollum is very carefully controlled and the results interpreted with reference to the amount of protein consumed as well as the level of intake, it is not an effective instrument of research. Since such precautions are not ordinarily taken by those using the method, the results obtained are very difficult to evaluate and the conclusions deduced cannot be intelligently criticised. The method has undoubtedly served a valuable purpose in revealing the dietary deficiencies of different kinds and classes of foods, but the tendency of the method very probably is to exaggerate such deficiencies, since in general rats will consume more of a more complete ration than of one less complete, and the difference in growth and other functions secured will therefore be the result of a larger intake of food as well as of a more favorable balance of nutrients. In interpreting the results of the method as it is ordinarily used with reference to the quality of food proteins, the frequent necessity of assuming a toxicity in natural foods or in the protein fraction itself (see 65 in particular), or the occasional appearance of results either unexplainable or explainable only on the assumption of an intricate interrelationship among the requirements of animals for different nutrients, hardly enhances the confidence which the conclusions can be accorded.

The method of expressing numerically the growth-promoting value of proteins. Throughout their work on the comparative nutritive value of different proteins and protein mixtures Osborne and Mendel have consistently recognized the great importance of food intake records, and have repeatedly pointed out that protein requirements cannot

[1] The fact that Osborne and Mendel (110), (112) have secured distinctly better growth with cereal proteins at an 8 or even a 5 per cent level than McCollum, Simmonds and Parsons (61), (65) at a 9 per cent level, using rations which, though not identical with the rations of the latter investigators, were closely comparable with them, can hardly be explained on any other grounds than a markedly better food consumption.

be expressed in, or be measured by, a minimum percentage of protein in a ration required for maintenance or maximum growth. They have also realized the tentative character of our present theories in regard to the necessary constituents of a complete diet and, provided successful and prolonged growth is obtained, have refused to attribute ultimate failures of growth and well-being to the character of the protein factor (112). By thus confining consideration in a given experiment to one particular type of performance, namely, growth, the attainment of strictly comparable figures expressing the nutritive value of proteins has been possible.

However, fair comparisons of the nutritive value of different proteins and mixtures of proteins are difficult to make, even though accurate food intake records are available, because an animal continuously increases its food intake during growth in response to continuously increasing requirements. Two rats, therefore, on two rations differing only in the quality of protein will eat different amounts of food in accordance with different rates of growth. The difference in the rate of growth is the resultant of two factors, a greater food consumption and a better quality of protein, and hence in itself would give an exaggerated idea of the difference in the quality of the proteins. If the attempt is made to restrict the food intake of the rat on the better ration to that on the poorer, it would probably still grow at a faster rate and would therefore be placed under a handicap. In this case the difference in the rate of growth would amount to an underestimation of the difference in the quality of the two proteins. Not knowing exactly how the energy requirements vary for different body weights, ages and degrees of activity, it would be difficult to so control the food intakes of the two rats that the difference in the rate of growth secured could be ascribed solely to the difference in the quality of the dietary proteins.

Osborne and Mendel have pointed out that comparisons of the value of different proteins for growth can be made when the level of protein intake is so adjusted that the animals eat the same amount of food and gain weight at the same rate, the protein factor thus being the only variable. Using this method they have reported (103) as careful a study of the nutritive values of different proteins for growth as can be found in the literature. As is evident from this brief statement of the essential conditions for success, this type of experiment is exceedingly difficult and laborious. In an attempt to obviate some of these difficulties, a method whereby the relative

values of proteins for growth may be expressed numerically was devised by Osborne, Mendel and Ferry (115).

With rations otherwise complete as judged by available information, and containing proportions of protein such that growth is restricted by the intake of this nutrient alone, rats will consume different amounts of food and grow at different rates, depending upon differences in physical activity and hence in energy requirements, differences in vitality, and differences in the inherent capacity to grow. This variation in results secured, however, may be largely reduced by computing the gains in body weight secured per gram of protein consumed. When this is done it was found that with rations containing increasing percentages of the same protein, the gain per gram of protein consumed increased to a maximum and, for higher percentages of protein, decreased progressively. The figure taken as the measure of the growth-promoting value of the protein is the *maximum* gain in weight per gram of protein consumed. Hence, in the form in which the method was originally proposed, the measurement of the nutritive value of a protein for growth can not be determined in a single series of experiments on one ration, but requires several series of experiments on rations containing varying percentages of the same protein in order to locate this maximum value.

This numerical method of expressing the growth-promoting value of proteins is capable of determining accurately which of two proteins is the better utilized in growth and has consequently been used in a large number of experiments designed to measure the protein value of different foods. The values obtained, however, have a comparative rather than an absolute significance, since they are to some extent dependent on the conditions under which the experiment is run.

Since the measure of growth taken is the increase in body weight, obviously it is essential that in comparative experiments the average increase in body weight should have approximately the same composition, since the nutritive requirements for gains of different composition may be presumed to be different. The composition of the gains put on by growing animals progressively changes with age, particularly with respect to the content of moisture, fat and protein. The best way of assuring the desired equality in the composition of gains other than the laborious method of analyzing the carcasses of control animals killed at the beginning of the test and of other animals killed at the end of the test, is to use animals of the same age, weight, and previous treatment and to conduct the experiment for as short periods

of time as may be required for an accurate measure of the actual gain in organized tissue. If the experiment is prolonged over several months with proteins differing greatly in nutritive value, the great disparity in the gains secured in the different groups of animals would probably indicate also gains of distinctly different composition, representing expenditures per gram of different amounts of protein and energy.

If the protein consumed by a growing animal is being used solely for growth, the gain secured per gram of protein consumed on a ration of definite protein content would be independent of the actual protein intake, provided that the intake was not more than sufficient to cover the requirements for growth. On the other hand, if a certain fraction of the dietary protein is being used to cover a maintenance requirement for protein proportional to the amount of protein tissue in the body, the gain secured per gram of protein consumed would tend to be greater the larger the intake of protein, connoting a larger proportion of the protein intake available for growth. Until recently it was generally believed that there is a basal catabolism of protein calling for a certain quota of dietary protein to maintain the integrity of the tissues. This view was consistent with, if not a corollary of, Folin's theory of an endogenous as well as an exogenous catabolism of protein. Recently, however, as the result of experiments demonstrating that incomplete proteins and even certain ammonium salts, can either spare body protein or diminish the catabolism of nitrogenous substances, theories have been proposed to account for a maintenance requirement for protein that are inconsistent with the conception of a constant basal catabolsim of protein (98, p. 329), (126, p. 105). These theories imply that, in a growing animal receiving an adequate supply of protein for growth, the endogenous catabolism of nitrogenous substances is either greatly diminished or reduced to an infinitesimal amount. The author has elsewhere discussed these theories (88) and has presented some indirect evidence that, in contradiction to them, there is a constant breakdown of tissue constituents containing nitrogen (85), uninfluenced, or but slightly influenced, by the absorption of dietary protein. Direct proof that a growing animal is using part of its protein intake to maintain the composition of its tissues is difficult if not impossible to obtain.

Regardless of theoretical speculations on this highly interesting subject, it has been the experience of the author in studying the results of nutrition experiments designed to assess the nutritive value

of food proteins in terms of gain per gram of protein consumed, that within a group of rats receiving the same ration the larger food intakes tend to be associated with the larger gains per gram of consumed protein. The correlation is not particularly close, since other disturbing factors are also operating, and is only clearly evident when the differences in food intake are wide. The existence of a maintenance requirement of protein proportional to the body weight of the rat very probably accounts largely for the decreasing gain per gram of protein consumed observed in successive periods, when the food intake is not increasing in proportion to increasing body weight (52), and for the lower protein index obtained in long feeding periods as compared with those obtained in short feeding periods when the relation of food intake to body weight is distinctly less in the longer than in the shorter observational periods (112).

In the second report of Osborne and Mendel on the nutritive value of the proteins of certain cereal grains (112) the widely different ratio between food intake and average body weight existing in the 4-week and 10-week feeding trials, particularly with barley and oat proteins, offers the opportunity of testing this explanation somewhat more completely. For each feeding period it is possible to represent the protein utilization by an equation such as the following: $ax + \frac{b}{y} = c$ in which a is the average weight of the rat for the period, b the average gain per week, c the average intake of protein per week, x the amount of protein required for maintenance per week per gram of body weight, and y the gain in weight per gram of protein consumed above maintenance. For each rat, there will be two such equations, one for a 4-week period and one for a 10-week period. On the assumption that x and y are the same in these two periods, it is possible to solve for these unknowns for each of the rats, by solving the two corresponding equations simultaneously. Confining consideration to the experiments with rations containing 5 per cent of protein, since this is closest to an actual maintenance level and the utilization of protein for maintenance would be the most likely to be the same as that on an actual maintenance ration, and to barley and oat proteins, since the difference in utilization of protein in the 4- and 10-week feeding periods was the greatest with these cereals, the maintenance requirement, x, for barley proteins was found to be 26, 22, 23 and 16 mgm. of protein per gram of rat per week, and for oat proteins, 21, 25, 14, 31 and 23 mgm. for those rats giving possible values. These values are quite comparable to those found for the proteins of wheat in actual maintenance trials (110), averaging 23 mgm., and the variation in these indirectly computed values is hardly greater than that actually found in the maintenance trials on wheat protein. The similarity in the nutritive value of the cereal proteins would lead one to expect closely similar figures for the weights of protein required for the maintenance of rats.

Gains consisting entirely of protoplasmic tissue would contain approximately 20 per cent of protein, so that, for a protein completely digestible and with a biological value of 100, the gain per gram of protein actually used for growth should be 5 grams. In later periods of growth, with gains containing less protein and more fat, even larger gains per gram of protein used for growth would be possible. The fact that with the most superior proteins, such as those of milk, the gains per gram of protein consumed rarely exceed 3 grams indicates rather clearly that the protein intake is being used in part for some other function than growth. That this other function may be the maintenance of the nitrogenous integrity of the tissues is indicated by the following computation. Lactalbumin fed at a level of 4.9 per cent, produced a gain of 2.36 grams per gram of protein consumed (115). Taking the requirement of this protein for maintenance at 12.5 mgm. per gram of rat per week (103), it may be computed that the gain on the 4.9 per cent ration per gram of protein consumed above maintenance was 4.47 grams, a figure approximating very closely to the value of 5 grams theoretically possible. At higher levels of protein intake, such a computation gives progressively decreasing values.

As originally presented, the method involved a comparison of different proteins based on the *maximum* gains per gram of protein consumed. As ordinarily used by other investigators, however, no systematic attempt is made to find this maximum value; in some cases comparisons are made at one level of intake only, and in some cases comparisons of different proteins are made at different levels of intake, quite arbitrarily chosen. The values obtained with different proteins apparently stand in quite different ratios to one another depending upon the level of protein intake. Thus, with rations containing 15 to 17 per cent of protein, lactalbumin and casein give closely similar gains per gram of protein consumed, while at an 8 to 9 per cent level, the value for lactalbumin is twice the value for casein (115); the maximum value obtained for lactalbumin was 3.01, and for casein 2.25. Similarly, a mixture of corn and rice bran proteins at an 8 per cent level gave distinctly greater gains per gram of protein consumed than rice bran proteins alone, while at a 9 per cent level, the efficiency of the mixed proteins was reduced and that of rice bran proteins was increased, so that the difference between the two was so much less as hardly to be significant (53). At present, it is hardly possible to judge whether the fairest comparison is given by the maximum gain per gram of protein consumed located by varying the level of protein intake, or

by the ratio of gain to protein intake at a definite level, not so high that in either case maximum growth is obtained. Obviously the index obtained at such levels of intake as to permit maximum growth cannot safely be taken as measuring the nutritive value of a protein.

It would seem, therefore, that a comparison of the nutritive values of different proteins and protein mixtures by the numerical method of Osborne, Mendel and Ferry, while capable of definitely establishing differences, does not give any certain basis for determining how much better one protein is than another. The gain in weight secured per gram of protein consumed, in other words, is not an absolute measure of the nutritive efficiency of the protein, since it is dependent upon the experimental conditions, particularly the size of animal, the level of protein intake, and the amount of food consumed relative to body weight. In fact, it is doubtful if the method is any more flexible than that originally proposed (103) in which, for rats of equal weight, conditions must be so adjusted that on equal amounts of food the rate of gain is the same.

In comparing the values obtained for different proteins by this numerical method, it should be remembered that they are affected by differences in the inherent digestibility of the proteins themselves and also by the presence of indigestible carbohydrates in the ration in so far as these substances depress the digestibility of the proteins.

Methods involving a study of the nitrogen metabolism. The gain in weight of an animal on a ration presumably deficient only in protein is a somewhat uncertain indication of protein retention, since it is of variable composition, and since it may be limited by deficiencies in growth determinants at present unknown. The vitiating effects of such unknown growth determinants would probably only appear in prolonged growth experiments leading to the exhaustion of the stores of such factors in the body. A more direct method, however, would seem to be the determination of the nitrogen balance, since it is generally believed that nitrogen is retained only as protein. Furthermore, the nitrogen balance in short periods of time does not seem to be affected by deficiencies of the diet in other indispensable nutrients (47), (25, p. 269); in fact McCollum and Steenbock (74),(54) have obtained satisfactory storage of nitrogen in pigs on inadequate diets. Until the body stores of these other nutrients become exhausted there is no reason to suspect that protein utilization would be interfered with, though, it should be mentioned, on such a suspicion McCollum has himself repudiated these early metabolism studies (55, p. 83).

A comparison of the nitrogen intake of an animal on a ration containing, as its sole source of nitrogen, the food whose protein value is to be determined, with the nitrogen balance resulting, has been used in measuring the nutritive value of food proteins. A percentage retention of the total nitrogen consumed is thus computed. While this computation may be a good *practical* measure of the net value of proteins from different sources in promoting growth, just as the ordinary coefficient of digestibility of a protein may be a good *practical* measure of digestibility, its value as a scientific criterion is impaired by the fact that it is difficult to interpret, since it is a resultant of several factors in digestion and metabolism, any one or all of which may be concerned in a particular difference in the percentage between two sources of protein. To obviate differences in the intestinal utilization of the dietary nitrogen, a percentage retention of the *absorbed* nitrogen (food N minus fecal N) may be computed and has been used in assessing the biological value of the food protein.

Both of these methods fall short of obtaining an absolute measure of biological value since, like the gain per gram of protein consumed, they neglect one purpose for which protein is being constantly used in the body, namely, the maintenance of the tissues. The tissues seem to be continually suffering a disintegration of nitrogenous substances, partly as the result of an endogenous catabolism within the cells, and probably in part, as Kestner (48) has recently pointed out, as the result of a constant synthesis of the protein constituents of the digestive juices, and their subsequent incomplete utilization in digestion and metabolism. By thus comparing intake with retention, the protein consumed is being credited with only part of its net value to the body. While this neglect does not greatly affect the values obtained for proteins of high biological value, since, even at a low level of intake, these proteins may occasion large retentions of nitrogen (54), it greatly undervaluates the cereal proteins, for example, so that the superiority of milk proteins in nutrition over cereal proteins is greatly exaggerated (see p.458).

The criterion of the biological value of a protein may be revised to meet this objection by expressing that fraction of the absorbed nitrogen that escapes excretion in the urine as a percentage of the absorbed nitrogen. The difficulty in determining such a criterion rests, first, in determining the actual absorbed nitrogen, due allowance being made for nitrogen of body or bacterial origin excreted in the feces, and second, in determining the urinary nitrogen of body origin.

Unfortunately there seems to be no direct method available for differentiating body from dietary nitrogen in either feces or urine, so that indirect methods, involving debatable assumptions, must be called upon. These corrections for body nitrogen may be made (85) on the basis of the nitrogen excretion in feces and urine of animals maintained on a nitrogen-free diet, consumed in amounts to cover the energy requirements for maintenance.

The method may be illustrated by the following analysis of an actual metabolism study (85) on a rat receiving a ration containing approximately 4 per cent of a mixture of proteins derived from corn and tankage—a packinghouse by-product used in farm rations. The rat consumed an average of 56.9 mgm. of nitrogen per day in a 7-day collection period. On this diet, the daily excretion of fecal nitrogen was 27.6 mgm., but of this nitrogen it is estimated from the fecal nitrogen excreted on a nitrogen-free diet, that 21.7 mgm. were derived from the body, leaving only 5.9 mgm. contained in the indigestible food protein. Hence, the rat was receiving daily 56.9 − 5.9 = 51.0 mgm. of absorbed nitrogen. During this period, the excretion of urinary nitrogen was 48.6 mgm. daily, of which 37.7 mgm. were the result of the catabolism of the body's own tissues (estimated from the excretion of urinary nitrogen on a nitrogen-free diet). Hence, only 48.6 − 37.7 = 10.9 mgm. of the absorbed dietary nitrogen were wasted in metabolism, and 51.0 − 10.9 = 40.1 mgm. were retained in the body. The biological value of the protein, therefore, is equal to 100 × (40.1 ÷ 51.0) = 79.

The biological value thus obtained is similar to that of Thomas (147), proposed for the analysis of metabolism data on adult men, though the reasoning upon which it is based seems more direct, and in particular does not involve the question of the precise significance of the so-called "metabolic nitrogen" of the feces.

Although this method seems to measure fairly the fraction of the absorbed nitrogen that is used by the body for all purposes, it cannot be considered an absolute value, characteristic for each protein and constant under all conditions. Obviously it will depend upon the proportion of the absorbed nitrogen that is used for growth, since the biological value for maintenance is different from that for growth. It will also depend upon the level of protein feeding (87). The search for a biological constant measuring the chemical adequacy of a protein in nutrition, such as Martin and Robison (51) conceive of, seems a vain one. The best that can be hoped for is the determination of values representing fairly the comparative worth of different proteins under certain controlled conditions; for the best results, the control should probably be as rigid as that specified by Osborne and Mendel

(103). How greatly the values thus obtained will vary with changing conditions is a subject for future research. Possibly the variation that would be met with under conditions of practical nutrition would be no greater than that to which coefficients of digestibility are subjected, so that average biological values for adult nutrition and for the nutrition of growing children may have considerable practical value in the computation of the net protein values of foods.

Is it possible to determine the biological value of proteins for growth or milk production? If the biological value of a protein were definitely fixed by its chemical constitution it should be possible to determine a value for growth as well as one for maintenance. The latter could be determined in a metabolism experiment in which all the dietary protein is being used for maintenance. The value for growth could be obtained from a metabolism test in which the food protein was being used for growth as well as maintenance, by correcting for the maintenance quota, in some such manner as Armsby (3, pp. 387–390) has used. Unfortunately, the situation is not as simple as this, since as the level of protein intake increases the efficiency with which it is utilized in anabolism decreases, as indicated clearly by nitrogen metabolism studies on rats (87). The variation of the gain in body weight of rats per gram of protein consumed in diets in which the protein factor limits growth (112), (115), (53), also testifies to the operation of other than chemical factors in determining the biological values of proteins. This decreasing utilization of protein nitrogen in anabolism is probably due to the fact that, at all levels of intake, some of the amino acids coming from the intestinal tract are subject to the oxidation processes in the cells, and that this percentage loss increases as the relative concentration of amino acids to non-nitrogenous nutrients accumulating in the cells during digestion increases in response to an increasing percentage of protein in the diet. In other words, the protein-sparing effect of non-nitrogenous nutrients is not completely effective at any level of protein intake (3, p. 322).

The increasing wastage of protein in oxidative catabolism as the protein intake increases from a maintenance level to one permitting growth would obviously concern as much the maintenance quota as the growth quota. For all practical purposes, it would amount to an increasing maintenance requirement for protein with increasing concentration of protein in the diet. As a consequence, if the calculations of the maintenance requirement of barley and oat proteins from the feeding experiments of Osborne and Mendel (p. 439) are made for

the rations containing 8 and 10 per cent of protein, much larger values are obtained than with the 5 per cent rations. The impossibility of correcting for the maintenance protein requirement in growth experiments, for the purpose of obtaining a biological value for growth alone, is thus evident. For the same reason it would be impossible, by any but indirect and approximate methods, to obtain a biological value of food proteins for the function of milk production. In such cases, composite biological values only, for maintenance and growth and for maintenance and milk production, can be determined.

Another obstacle in the path of any method of determining a separate biological value of protein for physiological functions, such as growth and lactation, necessarily superimposed upon the function of maintenance, relates to the very evident qualitative differences between the amino acid requirements for maintenance and growth, and very probably between those for maintenance and milk production.

In serving for the maintenance of the nitrogenous integrity of the tissues, any protein may be considered as consisting, under definite conditions, of two fractions, one destined to be utilized completely in anabolism, the other fraction, because of chemical inadequacy, destined to be deaminized and used in the body for other purposes in common with the non-nitrogenous nutrients. However, if a protein is serving simultaneously for maintenance and growth of the maintenance quota, the latter fraction, unsuited chemically for maintenance, may nevertheless be utilized in part at least by rendering more efficient for growth the remainder of the protein intake. In other words, the selection for maintenance of certain amino acids in certain proportions may leave a residual mixture of amino acids possessing a higher biological value for growth than the original protein, by reason of containing, for example, a greater concentration of lysine or cystine. Under such conditions, the impossibility of assessing accurately the value of a protein for growth alone from its determined value for maintenance alone and for maintenance and growth simultaneously, is obvious.

The determination of the biological value of proteins for maintenance. Since experimental conditions may be so controlled that all of the dietary protein is being used for the maintenance of the integrity of the tissues, it is possible to determine the biological value of proteins for this function alone. This may be done by determining the amount of protein from different sources required to maintain body weight, the ration being so constituted as to be adequate in all respects except for the concentration of protein (103), (110), (101). In nitrogen

balance studies, the same purpose is accomplished in a shorter time, with the further advantage of being able to assess the loss of protein in digestion, by determining the lowest level of protein intake consistent with nitrogen equilibrium (32), (126), (127), (128), (129), (119).

If comparisons between different proteins are made by these methods in closely adjacent periods and with the same subjects, or if simultaneous experiments are run upon a large number of animals, reliable comparisons are possible. However, the maintenance requirement of animals, as measured by the excretion of urinary nitrogen on a nitrogen-free diet, is variable from subject to subject, and for the same subject at different times (51), (85), (94), (89), (130), (2) in the same manner as the basal metabolism of energy and possibly for the same or analogous reasons. Hence, there may be some uncertainty in obtaining strictly comparable values for different proteins by determining at one time the lowest intake consistent with nitrogen equilibrium for one protein, and then at some distant date, making a similar determination for another protein on the same subject. If the purpose were to determine the effect of two proteins on the basal heat production, it would not be the best technic to make the two determinations at two widely different dates unless on each date the basal heat production is also ascertained.

Thomas (147) has proposed a method that avoids this objection, in that the endogenous loss of nitrogen is determined and the effect on the nitrogen balance of the ingestion of small amounts of protein from the different foods to be tested is measured in a closely adjacent period. In this way it is possible to compute how much digestible food nitrogen is required to replace a unit of body nitrogen degraded in the endogenous catabolism. The term "biological value" of protein was first applied by Thomas to the results of such a calculation. While the experimental work of Thomas has justly been criticised, the theory upon which his calculations were based seems essentially sound.

EXPERIMENTAL DETERMINATIONS OF THE NUTRITIVE VALUE OF PROTEINS. *The nutritive value of proteins for maintenance.* The requirements of the animal body for amino acids for growth are such that a shortage of any one amino acid limits the utilization of all the others. Certainly the main function of dietary amino acids in growth is to furnish the units or "building stones" for the construction of protein molecules. The requirement of the animal body for amino acids for the maintenance of the composition of its tissues, however, appears to be in quite a different category, in that, while certain amino acids are indispensable for the complete maintenance of the tissues, their absence

from the diet or their presence in inadequate concentration does not prevent the utilization of the other amino acids, though possibly this utilization is to some extent impaired. In this function, apparently, amino acids are serving as precursors of relatively simple nitrogenous constituents of the tissues (88). Whether the amino-acid requirements for maintenance are simpler than those for growth cannot at present be said; it is obvious, however, that some amino acids, such as lysine and cystine, are required in relatively greater amounts for growth than for maintenance.

The relative value of different proteins for maintenance has been investigated rather extensively on rats by McCollum and Simmonds (59), who have concluded that the cereal proteins are distinctly less efficient for this purpose than the proteins of milk, while the proteins of the flaxseed, navy beans, and peas are distinctly less efficient than those of the cereal grains. It is evident, however, from the charts illustrating the results of this experiment that maintenance of weight was secured in only a small minority of the animals used, and that the distinctions made between different sources of protein are often difficult to detect. In the absence of food records, the results secured are difficult to evaluate.

Using the criterion of continued maintenance of body weight, Osborne and Mendel have reported (101), (103) some studies on the relative values of lactalbumin, casein, edestin, gliadin and the mixed proteins of milk. Expressing the maintenance requirement as the smallest intake of protein per gram of rat per week consistent with constant body weight for six weeks or more when the energy intake was liberal, the average values obtained with uncontrolled food intake were 12 mgm. for lactalbumin, 19 mgm. for casein, 18 mgm. for edestin, 17 mgm. for the mixed proteins of milk, and 24 mgm. for gliadin. In a later experiment, in which the rats received a constant energy intake, the average values found for lactalbumin, casein and edestin, respectively, were 12.5 mgm., 16.4 mgm. and 15.0 mgm., and in another series of trials in which conditions were even more nearly identical, the average values obtained were 9.3 mgm., 15.5 mgm. and 13.8 mgm. Considerable variations were noted among individual rats, so that the significance of the average figures given above may not be great, while the interrelation between energy intake and protein minimum also prevents assigning a definite value to any one protein.[2] However, it seems

[2] These experiments suggest an interesting relation, hitherto uninvestigated as far as the author is aware, between the balance of nutrients in a ration and the utilization of its energy. The energy intake of these rats was liberal enough to support considerable growth when the protein concentration was increased.

clear that the lactalbumin ration was a better source of protein than either the casein or the edestin ration, while of the latter two, the edestin ration seemed slightly superior to the casein ration. The difficulty in interpreting these results in terms of the isolated proteins themselves lies in the fact that all rations used contained considerable amounts of nitrogen, relative to the nitrogen in the protein tested, in largely unknown forms in the "protein-free" milk, constituting about one-fourth of the rations. The extent, if any, of the supplementing effect of the nitrogenous compounds contained in this product prevents any definite interpretation of these otherwise excellent experiments.

This criticism does not apply, however, to the results of similar experiments on the value for maintenance of the proteins in the wheat kernel and its milling products (110), since no "protein-free" milk was used in compounding the rations. While the individual experiments were admittedly too few and too variable to permit the formulation of very definite conclusions, it seems evident that for the whole wheat kernel about 23 mgm. of protein per gram of rat per week were required for maintenance, for the wheat embryo somewhat less than 20 mgm., and for the proteins of the endosperm (wheat flour) about 23 mgm. or slightly more. It is noteworthy that for maintenance, the proteins of the wheat endosperm are about as efficient on this basis as the proteins of the entire kernel, though when allowance is made for differences in digestibility, the inferiority of the endosperm proteins would be more evident.

There are several metabolism experiments reported in the literature that indicate a practically complete utilization of the nitrogen of several proteins and protein mixtures. McCollum and Steenbock (74) have reported that the nitrogen excretion of the pig on a nitrogen-free diet is not appreciably changed when amounts of corn and oat protein are introduced into the ration (with a withdrawal of isodynamic amounts of starch) equivalent in nitrogen to but little more than the endogenous losses. With wheat proteins a small increase occurred, amounting to 10 or 15 per cent. Steck (130) has likewise shown in experiments on himself, that ingestion of small amounts of nitrogen in the form of meat, egg albumin and casein, equivalent to the endogenous loss, had no appreciable effect on the excretion of nitrogen in the urine; in fact, a much larger amount of meat nitrogen still had no such effect. Such results indicate a very complete utilization for maintenance of the nitrogen of these proteins and also of the non-

protein nitrogen of meat. However, hemoglobin in equivalent amounts caused marked increases in the urinary nitrogen, indicating a less complete utilization of the nitrogen of this protein. The author (85) has shown, in metabolism experiment on rats, that the ingestion of small amounts of lactalbumin and the mixed proteins of milk did not cause increases in the urinary nitrogen over that observed in periods of nitrogen-free nourishment.

Other experiments in which the protein intake is considerably larger in proportion to the endogenous losses, have revealed rather distinct differences in nutritive value among different proteins and protein mixtures in covering the maintenance requirements for protein. The most complete experimental investigation of this type that has ever been reported is that of Thomas (147). The results of Thomas are frequently quoted in textbooks and monographs on nutrition, obviously not because of their inherent accuracy and reliability, but apparently because they are quite comprehensive and have been very conveniently condensed into a tabulated summary of "biological values," representing the percentages of the absorbed nitrogen of each of the foods capable of replacing the nitrogen lost to the body in the course of the endogenous catabolism. The experiments were performed on Thomas himself. To the proteins of milk, meat and fish, Thomas assigns values approximating 100, indicating complete utilization of the absorbed nitrogen. On the other hand, to the proteins of corn and wheat have been given values of 30 and 40, respectively, while the proteins of rice, potatoes and peas are graded, in order, 88, 79 and 56. Thus, according to Thomas, animal proteins are two to three times more valuable in adult nutrition than the cereal proteins.

A study of the details of the experiments upon which these biological values are based, however, detracts greatly from their significance. The periods in which the different foods tested constituted practically the sole source of nitrogen were of one to four days' duration only, with no transition or preliminary periods. Apparently no attempt was made to insure a constant intake of nitrogen and calories during these short periods, nor were the different forms of nitrogen fed at comparable levels, considerable variations in calorie and protein intakes occurring from day to day throughout the experiments. Thus, the vitiating effect of the well-known lag in nitrogen excretion held full sway. The data are taken to indicate that protein nitrogen is better utilized when ingested in small portions several times a day than when ingested in one meal; however, this distributed intake was practiced

only with certain of the animal foods, i.e., meat, nutrose, milk, fish and crabs. Hence, the other foods are placed at a disadvantage, regardless of the metabolism data. Furthermore, in computing the average biological values for the proteins of the different foods, what seems to be a purely arbitrary selection of some days and rejection of others was practiced, resulting in an exaggeration of the difference in nutritive value between animal and vegetable proteins. As Hindhede has pointed out (32), the data are indeed so variable that by selecting certain days it may be shown that meat protein is about twice as valuable as wheat protein, while a selection of other days would lead to just the opposite conclusion. The selection of some experimental results in preference to others is always a hazardous undertaking.

In experiments on rats (87) the author has recently shown that when the protein constitutes but 5 per cent of the ration, permitting maintenance or only very slow growth, the proteins (nitrogen) of milk exhibit a biological value of 93, those of corn a value of 72, those of oats a value of 79, those of rice a value of 86, those of potato a value of 68, those of yeast a value of 85, while casein alone gave a value of only 71. These determinations do not indicate any extreme differences between the biological value of milk proteins and of vegetable proteins in adult nutrition.

Hindhede's experiments (31), (32) on men also indicate a good utilization of vegetable proteins in adult nutrition. On potato as the sole source of nitrogen, equilibrium was attained on a urinary excretion equivalent to 0.048 and 0.064 gram of nitrogen per kilogram of body weight; on rye bread as the sole source of nitrogen, equilibrium was attained on a urinary excretion of 0.047 and 0.050 gram of nitrogen per kgm. These values were obtained on 25 grams or less of digestible protein per 70 kgm. body weight, assuming all the nitrogen of the feces to be food nitrogen. Essentially the same results on potato nitrogen have been reported by Rose and Cooper (119) in this country, though in their experiment the digestible protein intake was somewhat higher, i.e., 29 grams per 70 kgm. body weight. The urinary nitrogen was equivalent to 0.060 gram per kgm. of weight. While Abderhalden and associates (1) have obtained results almost identical with those of Rose and Cooper for potato nitrogen, their results with the proteins of rye bread, in contradiction to the data of Hindhede, indicate a distinctly less efficiency. White bread proteins appeared to be no more efficient than rye bread proteins in this regard.

Sherman and his associates (126), (128), (129) have reported the results of metabolism experiments on human subjects demonstrating the attainment of nitrogen equilibrium on 35 to 40 grams of protein per 70 kgm. body weight, consisting to the extent of approximately 90 per cent of the proteins of cornmeal and oatmeal with 10 per cent of milk proteins, or consisting entirely of the proteins of white bread containing an undetermined proportion of milk proteins. According to Sherman, it does not appear to be necessary to discriminate against cereal proteins in the nutrition of adult human beings, since these proteins, with minimal additions of milk protein, seem to function in the metabolism of maintenance with an efficiency comparable with that of the protein of mixed diets.

However, the superiority of animal proteins alone in adult human nutrition is indicated in the work of Dienes (9), who has shown that in the human subject more favorable nitrogen balances were obtained on a meat diet than on a diet of wheat or peas, after extreme emaciation. Rose, MacLeod and Bisbey (121), in experiments on women subjects, have obtained as favorable nitrogen balances with soy bean proteins as with meat proteins when fed at a low level (0.08 gram N per kgm.), while milk proteins alone or combined with bread proteins (fed at approximately the same level) have given more favorable balances. In the latter experiments, of which only an abstract is available, the balances were all positive; hence the results may not apply solely to the maintenance requirement, since it cannot be assumed that, if the protein intake had been reduced to the point where negative nitrogen balances prevailed, the same relative differences would have been shown to the same degree.

While the experiments of Hindhede, Rose and Cooper, Abderhalden, Sherman, and Rose, MacLeod and Bisbey testify to the very low requirement of protein in adult human nutrition, and have been interpreted to indicate a very good utilization in metabolism of certain food proteins, the degree of utilization cannot be estimated in the absence of data on the endogenous losses of nitrogen. Indeed, if the endogenous loss in the urine may be taken as about 0.040 gram daily per kgm. of body weight, the utilization secured in several of these experiments could not have been better than 60 to 70 per cent.

Recently Martin and Robison (51) have attempted to measure the value of the proteins of milk as compared with those of whole wheat bread in replacing the nitrogen lost in the course of the endogenous catabolism. They determined for each of the two subjects the extent

of the endogenous loss of nitrogen in urine and feces by subsisting for a week or longer on a diet very nearly nitrogen-free. The effect on the nitrogen balance of the ingestion of increasing amounts of nitrogen in the form of the two foods to be tested was then determined, and the biological value calculated at each level of intake according to one of the formulas of Thomas. The minimum endogenous losses in the urine were found to be 38 and 35 mgm. daily per kgm. of body weight. The proteins of whole wheat bread were found to possess an average biological value of 35 and 31 for the two subjects. Nitrogen equilibrium was not attained until the intake was 10 to 12 grams daily, and the absorbed nitrogen 9.5 to 10.5 grams, the energy intake being 60 calories or more per kgm. body weight. This result is quite at variance with that of Sherman (126), i.e., nitrogen equilibrium on an intake of 6.0 grams of bread nitrogen for a man of 80 kgm. and an energy intake of 34 calories per kgm. The results with milk protein were extremely discordant and hardly explainable since, starting with nitrogen intakes approximating the endogenous losses, increasing amounts of milk nitrogen had no consistent effect on the negative balances secured until an intake of about 11 to 14 grams was reached, when equilibrium was indicated, the energy intake being 46 and 47 calories per kgm. On increasing the energy intake with one of the subjects to 57 calories per kgm., nitrogen equilibrium was approximately reached on 6.84 grams of nitrogen. The biological values calculated for one subject were so low that they were not considered in the final conclusions. With the other subject, an average value of 51 was obtained for those periods in which the increased energy intake prevailed. While these experiments were apparently well planned, well controlled and well executed, the results secured were so anomalous that one is at a loss to evaluate them.

In view of the contradiction existing among several of the experiments reviewed above, general statements of the relative nutritive values of food proteins in adult nutrition cannot be made with assurance. At levels of intake so low that equilibrium is not reached, animal proteins and even cereal proteins appear to be completely utilized in metabolism. For the attainment of nitrogen equilibrium however, different proteins appear to possess distinctly different efficiencies. This may possibly be due to the fact that a considerable fraction, possibly most of the nitrogen requirement for maintenance, is related to the replacement of relatively simple nitrogenous constituents of the tissues (88), and may be satisfied, therefore, by a less

complete assortment of amino acids than is required for growth, or, in part, even by ammonium salts. The attainment of nitrogen equilibrium, however, very probably means the satisfaction of the requirement for a certain amount of protein synthesis, particularly for the elaboration of the constituents of the digestive juices (48). While these proteins are in part subsequently digested and absorbed, losses of nitrogen in both feces and urine would seem to be inevitable. Hence, for the complete maintenance of the tissues, the body's demands for nitrogenous substances are in part quite diversified, perhaps even approximating in this respect the requirements for growth. The superiority of milk proteins in satisfying these requirements seems evident, though the cereal proteins are not greatly inferior to them. There is, quite evidently, a great need for more investigation of the value of different proteins in adult human nutrition; the general procedure of Martin and Robison would seem to be as effective as any in solving these problems.

The nutritive value of proteins for maintenance and growth. It seems impossible to obtain specific measurements of the value of different proteins for physiological functions, such as growth, reproduction and lactation, that must of necessity be superimposed upon the function of maintenance. The task of differentiating the fraction of the protein intake concerned with growth only, from that concerned with maintenance, so that the amount of growth secured or of protein added to the body can be related to that amount of dietary protein responsible for it, appears to be hopeless for reasons given fully in a preceding section. The alternative course, therefore, is either to relate the amount of growth secured to the total protein intake, or by proper metabolism experiments, to relate the amount of nitrogen apparently retained in the body for both maintenance and growth to the amount of dietary nitrogen apparently absorbed from the intestinal tract. The first method ignores the maintenance requirement entirely; the second gives a composite measure for both maintenance and growth.

Using their method for the biological analysis of foods in its most comprehensive form to include observations on growth, the maternal functions, the appearance of the characteristics of senility, and the well-being of succeeding generations, McCollum, Simmonds and Parsons (67) have arranged the proteins of a number of animal and vegetable foods in a series of decreasing biological value, as follows: 1, beef kidney; 2, wheat; 3, milk, beef liver; 4, beef muscle, barley, rye; 5, corn, oats; 6, soy beans, navy beans, peas. It will be observed that

the proteins of wheat are assigned a higher value than the proteins of milk or of meat in contradiction to earlier conclusions of the same authors (56), (61), while the proteins of barley and rye are placed on a par with the proteins of meat. These relations are quite contrary to those indicated by less ambitious methods of research. One naturally raises the question whether the method used by these investigators is equivalent to other methods hitherto considered adequate for the purpose. To the author there is little doubt but that growth experiments and nitrogen balance studies, conducted under proper control, can give reliable information of the chemical adequacy of proteins in nutrition. To account for failures in the proper performance of the maternal functions and for nutritive failures in the second and third generations, by reference solely to the source of protein in the diet, no matter how complete the diet may *seem* to be in other factors, is equivalent to assuming that present knowledge of nutritive requirements in complete. Such an assumption, however, cannot be justified. Since most of the factors involved in the final judgment of these comparative values of food proteins are not capable of numerical expression, and even the factor of growth is not so expressed, it is an extremely difficult matter, taking the results at their face value, to check the final conclusions, or to criticise them intelligently.

A large number of experiments have been reported concerning the nutritive adequacy for growth of the proteins of different foods and food materials. It has in general been found that most food proteins, if fed at a sufficiently high level, are capable of supporting normal growth in the rat. With certain leguminous seeds, however, namely, lima beans (13) and navy beans (37), practically no growth can be secured at levels of intake which induce normal growth with most food proteins; other leguminous seeds, the cow pea (15), the adzuki bean (40), and the vetch (135), also contain obviously inferior proteins, though the proteins of the common field pea (15) and the soy bean (145), (8), (107) are exceptionally good for seeds of this class. The proteins of the entire cereal seeds are adequate for normal growth at moderate levels of intake (110), (112), (145) with the possible exception of corn proteins, which do not seem to have been investigated under the most favorable conditions due to the low content of protein in corn. Hart and McCollum's work on the pig (23) demonstrates that corn supplemented with gluten feed, a protein concentrate prepared from corn, and minerals, is capable of supporting normal growth. The proteins of wheat flour seem to be definitely inferior to those of

wheat (110), (38); the evidence concerning the nutritive value of the endosperm proteins of corn is conflicting, Osborne and Mendel securing very little growth with such proteins (99), while Johns, Finks and Paul have secured normal growth with commercial corn gluten meal fed at a slightly higher level in a ration containing slightly less fat (42).

Experiments of the type just considered do not call for any extended comment, since they do not permit of any accurate comparisons of one protein or mixture of proteins with another, the rations used not being comparable in energy value and the actual intake of food not being recorded. It is interesting to note, however, occasional instances in which rations unsuitable for the support of growth or even maintenance were converted into rations suitable for growth or continued maintenance by the simple expedient of cooking (soy bean, cottonseed, cow pea, navy bean, lima bean), or the removal of toxic (velvet beans) or distasteful (pecan nuts, walnuts and almonds) ingredients. If these expedients had not been attempted, it is evident that a false idea of the inadequacy of the protein concerned would have been formed, simply due to the fact that the animals were not eating enough of the unmodified ration to constitute a fair test of its nutritive adequacy.

This method of the biological evaluation of food proteins is rendered somewhat more efficient by determining the growth of rats on different proteins fed at different levels of intake, no attempt being made, however, to relate the amount of growth secured to the amount of protein or food consumed. By this method McCollum and Davis (56) have shown that, with rations of comparable energy value (5 per cent added fat), 6 per cent of milk proteins was adequate for normal growth, while 6 per cent of the proteins of the whole wheat kernel gave very slow growth, comparable to that secured with only 4 per cent of milk proteins. Drummond (10) investigated the proteins of cod, herring and salmon muscle as compared with beef muscle proteins and casein, varying the proportion of each protein in diets containing 20 per cent of fat and noting the rate of growth secured. No differences were found in the growth-promoting value of the fish and beef muscle proteins, though casein seemed to be of slightly inferior value. Suzuki and his associates (145), (146) have confirmed these results of Drummond, indicating a similarity in the biological value of fish muscle proteins and beef proteins, and the distinct inferiority of casein. Normal growth was attained in these experiments on rations containing 10 per cent of animal proteins, 28 per cent of a modified "protein-

free" milk, and 14 per cent of fat. In these experiments,the extracted proteins of rice at a 10 per cent level gave excellent growth.

A further step in the refinement of biological methods for assessing the protein value of foods, relates the amount of growth secured to the intake of protein. By this method Osborne and Mendel (115) have demonstrated the superiority of lactalbumin over casein in promoting growth, when fed with protein-free milk, and the superiority of casein over edestin (103) under similar conditions of feeding. This latter finding is noteworthy, since in covering the maintenance requirement edestin has been found superior to casein (see above). In a later investigation on the nutritive value of the wheat kernel and its milling products (110), the same investigators obtained about 1.3 grams of gain per gram of protein consumed for the whole wheat kernel at a protein level of 10.3 per cent, a value of 0.5 gram for wheat endosperm proteins at a level of 14.7 per cent, a value of 1.6 gram for wheat embryo proteins at levels of 13.7 and 6.95 per cent, and for one rat a value of 2.05 gram for the proteins of wheat bran at a level of 9.05 per cent. If these values may be taken as entirely comparable—though they cannot be considered as maximum values since the effect of changing the level of protein intake was not sufficiently investigated—they indicate that embryo proteins and bran proteins are superior to those of the whole kernel, while endosperm proteins are markedly inferior. Since the endosperm contains almost three-fourths of the protein of the entire seed, it seems obvious that the bran and embryo proteins must supplement very efficienctly the proteins of the endo-sperm.

Their investigation of cereal proteins was later extended by Osborne and Mendel (112) to barley, oats and rye, as compared with wheat, using the whole kernel in each case. The maximum average gains per gram of protein secured with the different cereals, with the corresponding levels of intake, were: for barley, 1.89 at an 8 per cent level; for oats, 1.82 at a 5 per cent level; for rye, 1.49 at an 8 per cent level; and for wheat, 1.40 at a 10 per cent level. All of these values were obtained in periods of 4 weeks' duration. The differences indicated by these figures are not considered sufficiently significant by the investigators themselves to warrant any conclusion regarding the relative efficiency of the different cereal proteins. It is pointed out, however, that these values are not greatly less than those secured under comparable conditions for casein and lactalbumin.

Using the same numerical method of expressing the efficiency of proteins for growth, Morgan, Newbecker and Bridge (93) have made an interesting study of the proteins of the almond. While the mixed proteins of the almond were adequate for normal growth at a level of 17.2 per cent in rations containing 20 per cent of added fat, the globulin obtained by extracting the ether-extracted almond meal with 10 per cent sodium chloride, was distinctly less efficient; on the other hand, the fat-free residue after sodium chloride extraction and subsequent leaching with water to remove the excess of salt, contained proteins of exceptional efficiency. When fed at a level of 4.5 per cent in a ration the energy value of which is difficult to assess because of the indigestible character of much of the carbohydrate matter in the nut residue, these proteins produced almost normal growth with a ratio of gain to intake markedly higher than that secured with the almond meal. For the rats fed on this ration, an average gain of 3.87 grams per gram of protein consumed was secured in a 4-week period. This exceeds any value thus far reported by this method of expressing protein efficiency.[3] It is interesting to note that, in a comparable investigation of the proteins of the English walnut, Mignon (81) has not discovered any clear distinction in growth-promoting value among the fractional proteins of this nut.

Passing next to investigations of the proteins for maintenance and growth involving a study of the nitrogen metabolism of the experimental animals, the work of McCollum (54), comparing the proteins of the cereal grains with those of milk in promoting nitrogen retention in the pig, is worthy of special comment. McCollum found that, of the total nitrogen intake, about one-fourth was retained for growth in the case of corn, wheat and oats; of the absorbed nitrogen from the same food materials (in the calculation of which an estimate of the "metabolic nitrogen" of the feces was used) approximately 30 per cent was

[3] The opinion expressed by the authors of this report (p. 185) that "unless normal growth is attained during the period of observation a numerical expression of protein potency cannot be found by the process of dividing the total protein intake by the gain in body weight" cannot be subscribed to, since when normal growth is secured the protein content evidently is not a factor limiting growth, and the possibility that an excess of protein above the requirements for maintenance and growth is being consumed, renders the ratio obtained of doubtful value as a measure of the biological adequacy of the particular protein under investigation. This situation is clearly recognized by Maynard in his use of the method (53, p. 147). Contrary to Morgan, Newbecker and Bridge, Maynard discards all the results of feeding trials in which normal growth was secured.

retained. The data of the individual experiments do not offer any clear evidence of differences in the utilization of the nitrogen of the three cereal grains tested. In one experiment on casein, 46 per cent of the nitrogen intake, equivalent to 51 per cent of the absorbed nitrogen, was represented in the nitrogen balance. In one experiment on skim milk, these percentages were 63 and 66, respectively. However, when the biological efficiency of a protein is computed in such a way as to neglect the nitrogen used for maintenance, a false impression is conveyed of the differences existing in this respect among different proteins. From such computations the conclusion might be drawn that casein and the mixed proteins of milk are two to three times more efficient than the proteins of the cereal grains. Since the creatinine nitrogen excreted in the urine was determined in these experiments, it is possible to compute the total endogenous nitrogen on the assumption that this is 5.5 times the creatinine nitrogen (74), and to calculate biological values involving all of the purposes for which nitrogen was used (85). The results of such a calculation give average values for corn, wheat and oat proteins of 48, 44 and 42, respectively, the value for casein being 67, and for milk proteins 80. These figures are percentages of the absorbed nitrogen presumably used for maintenance as well as growth; while the marked superiority in nutrition of the mixed proteins of milk is still evident, they cannot be said to be as much as twice as valuable as the cereal proteins. Casein itself does not seem to be greatly superior to the proteins of corn, wheat and oats. In fact, in two earlier experiments on pigs reported by McCollum and Steenbock (74), the percentage efficiency of casein computed in a similar manner is only 57 and 58.

The author (87) has recently reported a series of nitrogen balance studies on rats designed to determine the biological value for maintenance and growth of different food proteins when fed at a 10 per cent level in rations containing 10 per cent of added fat.[4] The percentage

[4] Unfortunately no explicit information is given in this paper concerning the composition of the rations used. The general procedure used in our laboratory, however, has been explained in earlier papers appearing in the Journal of Dairy Science (94), (89). We have followed the procedure adopted by Osborne and Mendel in this respect, of supplementing the foods to be tested with butter-fat (vitamin A), the Osborne and Mendel salt mixture, and adjusting the protein level by the inclusion of varying amounts of starch, subsequently dextrinized by steam cooking. For those rations containing 40 per cent or more of a natural food product, no source of vitamin B was added. In other rations, small amounts of the Osborne and Wakeman fraction II from yeast have been used as a source of vitamin B, in amounts contributing from 1 to 2 mgm. of nitrogen per 10 grams of ration.

values obtained are entirely comparable with those computed from the metabolism work of McCollum. For the mixed proteins of milk an average efficiency of 85 was found, for corn proteins 60, for oat proteins 65, for potato proteins (N × 6.25) 67, for navy bean proteins 38. In earlier papers from the Division of Animal Nutrition at the University of Illinois (94), (89), values have been reported for the proteins of rice bran, 68, cottonseed, 66, soybean, 64, alfalfa hay, 62, and coconut, 58. From unpublished experiments we have found average biological values at an 8 per cent level of intake of 93 for the proteins of the hen's egg, 67 for the proteins of wheat, and 74 for the proteins of pork muscle. The differences among the biological values of the protein from these various classes of foods, with the exception of navy beans, are not extreme, as many statements in the literature would lead one to expect; no greater, for example, than differences in their digestibility. In particular, the nitrogen of meat does not seem to be much more completely utilized in metabolism than the nitrogen of whole wheat. However, the evidence is clear that the proteins of white flour and of many legume seeds are markedly inferior in their biological efficiency for growth.

Do the biological values of proteins for different animals differ greatly? Any consideration of the general significance of the biological values of proteins here discussed must include the question whether different species of animals exhibit different efficiencies in their utilization of protein in anabolism. There are some data on rats and pigs for a few foods and combinations of foods that permit a comparison of these two species. The biological values we have obtained for the proteins of corn at a 10 per cent level of intake average about 58, with individual variations ranging from 46 to 69. From the Wisconsin data on pigs we have already quoted an average value of 48 for five pigs. From another publication by Hart and Steenbock (26) designed to test the supplementary relations between the proteins of corn and those of milk, it is possible to compute[5] the biological values of the different mixtures of proteins, using average figures previously obtained at the

[5] In all of the calculations of the biological values of protein from the Wisconsin metabolism data on swine, we have assumed the following factors for the endogenous nitrogen losses in the urine for pigs of different weight: 40 to 65 lbs., 2.30 grams per 100 lbs.; 65 to 75 lbs., 2.25 grams; 75 to 85 lbs., 2.20 grams.; 85 to 95 lbs., 2.10 grams; and 95 to 105 lbs., 2.00 grams. These factors have been selected from various Wisconsin publications on the nitrogen excretion of pigs maintained on a ration of starch and salts.

same laboratory for the endogenous nitrogen of pigs. These computations are included in table 2 of this review. It will be seen that for the mixture of corn proteins 96.8 and milk proteins 3.2 the biological value is 64, while for the mixture of corn proteins 93.6 and milk proteins 6.4 the value is 66; it, therefore, seems fair to assume that, in this experiment with rations containing about 9 per cent of protein, the efficiency of the corn proteins alone was about 60. It may perhaps be concluded, therefore, that rats and swine utilize the absorbable proteins of corn in anabolism with about the same efficiency.

We have some analogous experiments on chickens relating to corn proteins. These data are summarized and analyzed in table 1.

TABLE 1

The biological value of the proteins of corn for chickens

HEN	AVERAGE WEIGHT	DAILY FOOD INTAKE	DAILY NITROGEN INTAKE	DAILY NITROGEN EXCRETION	NITROGEN EXCRETION ON N-FREE RATION	BIOLOGICAL VALUE OF PROTEIN
	grams	*grams*	*mgm.*	*mgm.*	*mgm.*	*per cent*
129	2126	44	717	613	298	56
129	2269	50	815	715	295	56
130	1610	44	717	624	338	60
132	1407	42	685	545	295	62
135	1732	44	717	606	260	52
74	2144	50	815	715	300	49
85	2243	75	1223	992	292	43
151	2492	75	1223	862	249	50
Average						54

The metabolism data were all obtained in collection periods of 7 days preceded by several weeks of corn feeding. The ration was coarsely ground yellow corn either alone or supplemented by 2 grams of a mineral mixture per bird daily. Since no attempt was made to separate the urinary from the intestinal excretions the calculation of biological values was necessarily different from that used for rats and swine, and may be best explained by illustration. For hen 129, the food nitrogen excreted was taken as 613 − 298 = 315 mgm., and that used in the body for anabolic purposes as 717 − 315 = 402 mgm. Assuming the food nitrogen to be totally digestible, the biological value, therefore, is (402 ÷ 717) × 100 = 56. That this assumption is not greatly in error appears probable from the fact that the true digestibility of corn proteins by the rat seems to be about 95 per cent

(85). Assuming this degree of digestibility in the hen, raises the biological value about 3 points, so that the average for all hens, instead of being 54, would become 57. It thus appears probable that the hen utilizes the nitrogen of corn in anabolism with about the same efficiency as the rat and the pig.

In this connection it should be mentioned that the biological values the author has reported for oat and wheat proteins for rats are considerably higher than those computed from McCollum's metabolism data on swine. However, our value for milk proteins at a 10 per cent level, 85, checks reasonably well with the computation from McCollum's experiment in which the milk proteins constituted 15.5 per cent of the ration, the biological value in this case being 80. The comparison between the two species can be extended a little further (83). Nevens (94) has reported a biological value of 58 for the rat for a mixture of corn and alfalfa proteins in equal proportions; Hart and Steenbock (25) have published metabolism data on swine, from which it may be computed, by the use of average factors for the endogenous nitrogen losses of the pig, that a mixture of corn and alfalfa proteins in the proportion of 64 to 36, has a biological value of 63. Similarly, a mixture of corn and milk proteins in the proportion of 3 to 1 has a biological value of 76 for the rat (86) and a value of 80 for the pig. Corn and tankage proteins in equal proportions have a biological value of 65 for the rat, and, in approximately equal proportions, a value of 68 for the pig.

The preponderance of available evidence seems to favor the view that different species utilize proteins in metabolism with about equal efficiency.

The relation of the amino acid constitution to the biological values of proteins. It is a perfectly obvious proposition that the biological value of a protein is primarily limited by the proportions in which the amino acids that cannot be synthesized by an animal occur in its molecule. This chemical limitation is such that for each protein or mixture of proteins a deficiency in one of the indispensable amino acids curtails its value for structural purposes in the animal body. When this deficiency is remedied by the addition of the appropriate amino acid, a deficiency of some other amino acid may constitute a second limiting factor, and so forth.

Considerable success has, in some instances, rewarded attempts to relate the biological inferiority of certain proteins with amino-acid deficiencies. The deficiency of zein in tryptophane and lysine (98),

of gliadin in lysine (97), of casein in cystine (100), (103), and of edestin in lysine (100), has been clearly indicated by feeding experiments with rats reported by Osborne and Mendel. Hogan (33) has shown that the first limiting chemical factor in the utilization in metabolism of the mixed proteins of corn is tryptophane and the second limiting factor is lysine. While this experiment would be more conclusive if the intake of protein had been recorded and controlled, the responses to amino acid additions and withdrawals seem sufficiently prompt and distinct to warrant the interpretation given.

A very conclusive demonstration that the mixed proteins of beans of the genus Phaseolus are deficient in cystine and that the addition of this amino acid has a marked effect in improving their biological values, has been furnished by Johns and Finks for the navy bean (37), and particularly for its main globulin, phaseolin, for the adzuki bean (40) and for the lima bean (13).

For such demonstrations, records of food intake are almost essential. If the difference in the growth secured with the unsupplemented and the supplemented proteins is constant and marked, the obvious conclusion has been established with a high degree of probability, but the final proof must rest in the demonstration that the gain per gram of protein consumed is greatly increased, out of all proportion to the increased food intake, or better yet, that on equal food intakes the addition of the supplementing amino acids has a pronounced effect in increasing the gains secured. The supplementing effect of amino acids may also be clearly demonstrated by suitable metabolism studies (50), (87).

Many experiments may be found in the literature purporting to show that certain proteins are deficient in certain amino acids, when, as a matter of fact, the results defy intelligent interpretation, because food intake records are not reported, and the differences in the rate of growth on unsupplemented and supplemented rations are neither invariably secured nor particularly marked when they do appear. This criticism applies with particular force to experiments in which the unsupplemented protein is fed for a time, and then a change is made to another ration in which small amounts of amino-acid additions are incorporated. A slight response in growth may result in such a case simply because the offering of a freshly made ration may induce a greater intake of food; or possibly certain labile constituents of the first ration may have undergone deterioration in the course of time with a consequent slowing of growth in the experimental animals, and

a prompt response in growth when a fresh ration adequate in these unknown constituents is available.[6] When responses to amino-acid additions in experiments of this type are not secured in all cases, the plea that this may have been due to irreparable injury caused by previous amino-acid deficiencies adds nothing to the argument. The mere fact that so many possible explanations may account for the results secured is sufficient proof that experimental conditions were not sufficiently described nor controlled.

It is unfortunately necessary to emphasize in a review of this character that certain biological methods of experimentation inherently faulty and inefficient are being extensively used in the investigation of many problems connected with the nutritive value of proteins. The inevitable result has been that confusion prevails where orderly and consistent results might have been obtained with the expenditure of but little additional effort. The problem whether lactalbumin is a complete protein or a protein of superior biological value, only recently settled by Osborne and Mendel (114) in the affirmative, is but an illustration of a case where the introduction of incompetent evidence has confused the issue. Perhaps, more than any other phase of the subject of this review, that of the supplementary relations existing among proteins has suffered the most from the employment of methods of research so designed that a clear-cut interpretation of their results is impossible.

The supplementary relations among proteins. It has been suggested that, for the purposes of practical dietetics, the different foods be classified according to the quality of their proteins into class A, B, C, etc. The difficulty encountered by this system is that the biological value of the proteins in mixtures of foods, such as are used in practical dietetics, is not necessarily the weighted mean of the biological values of the proteins of the individual foods. We may consider each food protein fed at a low level of intake as consisting of two fractions, one including the maximum amount of the several amino acids that can be used to replenish or enlarge the supply of nitrogenous substances

[6] See Steenbock, Hart, Sell and Jones, Journ. Biol. Chem., 1923, lvi, 385. The following quotation is of interest in this connection: "We desire to call attention to the irregularity of growth on the basal ration which we were at a loss to understand until we looked up our data on consumption. We then found that each time that the animals gained weight the ration had been made up fresh. Apparently the cod liver oil of the ration had undergone deterioration in vitamin content with aging. Of the deterioration there can be no question. . . . ."

in the tissues, the other including the remaining amount of the constituent amino acids destined to be deaminized, because it does not contain the complete assortment of amino acids essential for synthesis into complexes needed by the tissues. If the two foods are fed together to a growing animal, those fractions of each that would otherwise be deaminized may together contain a complete assortment of amino acids, permitting a part of the combined fractions to be used for synthetic purposes. In such a case, obviously the biological value of the mixture would be greater than the weighted mean of the biological values of each.

In demonstrating a true supplementary relation between two proteins or between the mixed proteins of two foods, it seems essential to measure the biological value of each protein or of the mixed proteins of each food separately, and also in combination, the level of protein intake in all three tests being the same. Obviously, such a demonstration requires quantitative methods relating protein intake to growth secured, nitrogen absorbed to total nitrogen retention, or some equivalent relation. The mere inspection of curves of growth obtained on rations limited only by their content of the individual proteins and of the mixed proteins is not sufficient to prove a supplementary relation, though it may establish a certain probability for the existence of such a relation. Furthermore, the proof by quantitative methods that the substitution of small amounts of proteins inadequate for normal growth at a given level of protein intake by equal amounts of proteins of superior biological value, enhances greatly the growth-promoting value of the ration, is not a clear demonstration of a sup lementing effect.

Maynard, Fronda and Chen (53) have demonstrated a supplementary relation between the proteins of corn and of rice bran. At an 8 per cent level, the gain per gram of protein consumed for corn proteins alone was 1.00 gram, for rice bran proteins alone 1.12 grams and for a mixture of the two in the proportion of 2 to 1, 1.71 grams. At a 9 per cent level of intake, the values were, respectively, 1.18, 1.47 and 1.63 grams. In this case, the mixture of proteins was evidently more efficient than the proteins of either food alone.

Using the data obtained from nitrogen metabolism studies on pigs, Hart and Steenbock (26) have investigated the protein efficiency of mixtures of corn and milk in various proportions, with results summarized in table 2.

The marked rise in the percentage of the nitrogen intake retained by the pigs as the proportion of milk protein is increased from 20.7 to 28.5 per cent is due largely to a marked improvement in the digestibility of the nitrogen of the ration. A less marked increase is indicated by the percentages of absorbed nitrogen retained, while the percentages of absorbed nitrogen used for both maintenance and growth (the biological values) show a linear increase from the first to the last group of pigs, with the exception of the next to last group on a ration containing 20.7 per cent of milk nitrogen. From the computations in the last column it is apparent that if corn had been fed alone

TABLE 2

The biological efficiency of the mixed proteins of corn and milk fed in different proportions

(From published data of E. B. Hart and H. Steenbock)

PERCENTAGE OF CORN AND MILK NITROGEN IN RATION		PER CENT PROTEIN IN RATION	NITROGEN BALANCE IN PER CENT OF		PER CENT OF ABSORBED N USED FOR MAINTENANCE AND GROWTH*
Corn	Milk		The nitrogen intake	The absorbed nitrogen	
96.8	3.2	8.6	21	40	64
93.6	6.4	8.9	22	40	66
88.3	11.7	9.4	25	46	72
79.3	20.7	10.2	37	56	73
71.5	28.5	10.9	60	73	87

* These values have been calculated by the author from average losses of endogenous nitrogen by pigs reported previously by Wisconsin investigators. See footnote 5.

its proteins would have shown an efficiency of a little over 60 per cent. With the fifth group of pigs receiving about 30 per cent of milk proteins, if no supplementary relation existed and if the efficiency of the milk proteins is taken as 100 per cent, it may be computed that the efficiency of the combined proteins in this ration would be about 72 per cent. The difference between 72 and 87, the efficiency actually attained, demonstrates a supplementary relation between the proteins of the two feeds.

The author (86) has investigated the supplementary relations between corn and milk proteins in the proportion of 3 to 1 at a 10 per cent level of intake, using rats as subjects. Corn proteins alone exhibited an average biological value of 61.3 in these experiments, milk proteins alone a value of 84.7, and the combination a value of 75.7. The mean of the first two values weighted 3 and 1, respectively, is

67.2. Therefore, the difference between this mean and the value actually found, 75.7, indicates clearly that the amino-acid deficiencies of corn proteins are supplemented by the excess amino acids of milk proteins.

While several instances of supplementary relations between animal and vegetable proteins have been indicated (110), (105), (108) or demonstrated by feeding experiments or metabolism studies, few instances of an appreciable supplementary effect of the proteins of one vegetable product upon those of another have been reported, insofar as the author is aware. Nevens has reported from this laboratory the results of combining in all possible ways the proteins of corn, cottonseed meal and alfalfa (94); for only one combination, i.e., that of cottonseed meal and alfalfa, was any such effect indicated, and this effect was slight. In another report (89) we have shown that no supplementary relations exist between the proteins of cocoanut meal and rice bran, nor between the proteins of corn and cocoanut meal, though between the proteins of corn and soy beans there may be a slight supplementary effect. When it is considered in this connection that on the average some 43 per cent of the protein of the American diet is derived from milled cereals, and almost 9 per cent from legumes, making 52 per cent of proteins of exceptionally low biological value for growth, the importance of animal proteins in the diet is obvious, particularly for children and convalescents. It is perhaps no exaggeration to say that the importance of animal proteins in the diet resides as much in their capacity of supplementing cereal and legume proteins as in their own excellence.

The statement is occasionally encountered in the literature that small proportions of proteins may exhibit a remarkable supplementary effect on the main protein fraction of a ration, and has been urged particularly against the validity of experiments to measure the nutritive values of proteins by the use of rations containing small amounts of nitrogen of unknown value in the materials introduced for their content of vitamin B. However, the author is not aware of any experimental evidence in support of this assertion. Certainly the metabolism data of Hart and Steenbock relating to the supplementary relation between the proteins of corn and milk do not indicate any marked effect of small additions of milk proteins, though milk proteins undoubtedly have a marked supplementary effect on corn proteins. Much has been said, for example, of the supplementary effect of the nitrogenous compounds of Osborne and Mendel's "protein-free" milk, but, on critical examination, nothing has been proved concerning it. It is obviously advisable to reduce the proportion of such foreign nitrogen to the lowest possible figure; however, the vitiating effect of such amounts as may be necessary is frequently exaggerated. It should be realized

that, in supplementing proteins with pure amino acids to detect their chemical deficiencies, additions of 2 to 3 per cent (122), (102) seem to be essential. If the foreign nitrogen amounts to only 2 or 3 per cent of the total nitrogen of the ration and is contained in an assortment of amino acids as well as other nitrogenous compounds, any marked supplementing effect on the protein under examination is extremely improbable. The Osborne and Wakeman fraction from yeast (yeast vitamine-Harris B), for example, has been found in our laboratory to contain 9.35 per cent of total nitrogen of which 16 per cent is free amino nitrogen and 28 per cent is combined amino nitrogen. As a source of vitamin B in the amounts required by rats (20 to 40 mgm. per day), it cannot reasonably be considered as affecting to any appreciable extent the biological values of proteins obtained.

The biological value of proteins for milk production. It would seem that the most profitable experimentation on the relative values of different proteins for milk production must be confined to those species of animals from which the milk can be collected, measured, and analyzed. Otherwise the milk production must be measured by the growth of the young, which would be affected by variations in the composition of the milk as well as variations in the rate of its secretion. The interpretation of the growth secured in terms of the amount of protein consumed by the mother would be difficult in such a case.

The work of Hart and Humphrey on the effect of the quality of food proteins on milk production in dairy cows illustrates well the difficulties involved in this type of research, even when the milk can be collected, measured, and analyzed. With immature or pregnant cows, the efficiency of the protein intake, however measured, relates to maintenance, growth, and milk production. If the cows are in negative nitrogen equilibrium, tissue proteins are undoubtedly being used for milk production and are, in all probability, supplementing the food proteins for that purpose. If considerable positive nitrogen balances are obtained, there is no assurance but that protein is being consumed in excess, thus depressing any calculated efficiency of the food proteins, particularly of the most superior combinations. It is significant that in the only experiment of Hart and Humphrey in which positive balances were obtained generally (21), the differences between the calculated efficiencies of the protein of the feeds tested were least.

While the experiments of Hart and Humphrey on dairy cows, and of Hoobler (36) on women, indicate the superior value of milk proteins for milk production, they also indicate that the difficulties attending this type of investigation have not been removed sufficiently to permit the calculation of protein efficiencies that can readily be interpreted.

The physiological effects of protein. Any complete survey of the nutritive value of proteins must include some consideration of the conclusion so frequently expressed, that protein consumed in amounts much above the actual requirements of the body, may exert harmful physiological effects. In support of this conclusion, reference is often made to the well-known work of Chittenden on the low protein dietary. However, in these classical experiments, the absence of control groups maintained upon the usual level of protein, renders questionable the deduction that the observed benefits of the low protein dietaries adopted were due solely if at all to the reduction in protein intake. In fact, the recent success of McCollum, Simmonds and Parsons (63, p. 126) and of Osborne and Mendel[7] in raising rats on diets containing 70 and 90 per cent of protein respectively, certainly is a strong argument against ascribing to protein deleterious physiological effects when fed even in great excess. Several instances in recorded human experience may be cited to the same purpose. However while it is probably true that protein may be indulged in to great excess without any immediate ill effects, or even with no pronounced ill effects at all, the wisdom of so doing may be questioned. The comfort of an individual, as well as his mental and physical efficiency are undoubtedly adversely affected under certain conditions by a high-protein dietary. The degree and type of activity of the individual should be considered, and the protein intake graded in proportion to the muscular activity. The well-known stimulating action of protein foods on metabolism probably is related to vitality and stamina, and should be numbered among the favorable effects of protein as a nutrient. At the same time the pronounced heating effect of protein associated with its stimulating action on metabolism, will naturally and rightly lead to a seasonal variation in the popularity of protein-rich foods.

It has been claimed that pellagra is a disease due to amino-acid deficiencies in the same way that beriberi, scurvy, and rickets are due to vitamin deficiencies. Since pellagra, or a disease of similar etiology, has not been induced in experimental animals, arguments concerning the cause of pellagra have largely been based upon statistical observations of the diet of pellagrins as compared with that of non-pellagrins under the same hygienic and other environmental conditions. Such observations, supplemented with a certain amount of quasi-experi-

[7] Osborne and Mendel, "Feeding experiments with mixtures of foodstuffs in unusual proportions." Proc. Nat. Acad. Sci., 1921, vii, 157.

mentation, such as one must be satisfied with in investigating the pathogenesis of deficiency diseases in human beings, has established clearly that dietetic error or errors of some description are primarily responsible for pellagra, and that the presence in the diet of certain amounts of protein-rich animal foods, particularly milk and meat, is an effective preventive against this disease.[8] However, the disease has not been definitely traced to protein or amino-acid deficiency, even though the arguments of the proponents of this deficiency theory are granted *in toto*. Thus, while Goldberger and Tanner[9] have recently shown that neither mineral supplements to a pellagrous diet nor additions of sources of the known vitamins, exert a protective action against the onset of pellagra, the dietary factor in the causation of the disease is not by these observations necessarily identified with the character of the protein supply. The conclusion to this effect is based upon the assumption that all dietary factors essential to the physiological well-being of humans are known; such an assumption is not justified by the facts, though it is commonly made. In considering the evidence of Goldberger and Tanner the omission of a direct attempt to show that the addition of adequate amounts of a good protein to a pellagrous diet would protect against the disease, is keenly felt.

Against the conclusion that "the dominating rôle of diet in the prevention and causation of pellagra must be referred primarily to the character of the protein (amino acid) supply," may be cited the fact that it is inconsistent with the statistics of the age incidence of the disease. The highest incidence is among adults, whose amino-acid requirements are less intense than those of adolescents and whose utilization of inferior food protein is better than that during growth. Thus, the proteins of white flour are markedly inferior to those of most other foods for growth, but much less if at all inferior for maintenance. Direct and unequivocal experimental evidence is essential before it can be concluded that amino-acid deficiencies are primarily responsible for pellagra.

[8] See Goldberger, Wheeler and Sydenstricker, "A study of the relation of diet to pellagra incidence in seven textile-mill communities of South Carolina in 1916. U. S. Public Health Repts., 1920, xxxv, 648.

[9] Goldberger and Tanner, "Amino-acid deficiency probably the primary etiological factor in pellagra." Public Health Repts., 1922, xxxvii, 462.

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THE MECHANISM OF VOMITING

ROBERT A. HATCHER

Department of Pharmacology of Cornell University Medical College, New York City

1. THE RÔLE OF THE STOMACH. Magnus (35) in 1903, reviewed the literature relating to vomiting but many of his conclusions are no longer tenable and many papers that have appeared since then show the influence of prevalent errors. It is necessary, therefore, to reconsider some of the older literature, and some of the more recent may be disregarded or treated briefly.

The starfish (asterias) empties the stomach through the mouth, the only opening into the stomach, and thus rids itself of useless, inert and indigestible material (52); the frog is capable of vomiting by the action of the stomach alone without aid from other structures (37); man vomits through the coördination of numerous reflexes. There are many gradations from the process in asterias to the type of vomiting usually seen in man, and much of the misunderstanding concerning the actions of emetics and the mechanism of vomiting has resulted from a too broad application of the results observed in a single species or in a few closely related species.

The mechanism of vomiting varies somewhat with the character of the gastric contents. Fluid may be expelled from the mammalian stomach by a primitive mechanism, but the expulsion of a pasty mass requires the aid of the accessory muscles. Obviously, the mechanism of vomiting varies with experimental conditions, and no single experiment suffices to explain the rôle of the various structures concerned. The present discussion is concerned mainly with the mechanism of vomiting in the cat, dog and man.

Cannon (3) states that Wepfer (56) in 1679, observed the closure of the pylorus and contraction of the pyloric part of the stomach during vomiting in cats, dogs and wolves but the question whether the stomach participates in the act of vomiting, and if so, to what degree, has been the subject of continued dispute. Magendie (34) contributed to the confusion on this subject by his celebrated experi-

ment in which he replaced a dog's stomach with a pig's bladder after which he induced vomiting. That experiment throws little light on the mechanism of vomiting because the cardia was removed with the stomach.

One of the members of the committee (Cuvier, Pinel and Humboldt) that reported on Magendie's memoir stated that he had seen a soldier vomit after partial recovery from an injury in which a bullet had severed the abdominal muscles so that they could take no part in vomiting at a time when the stomach was in full view. Doctor Gold in this laboratory (results not published) caused vomiting in a cat in which the abdominal muscles had been severed longitudinally and transversely, so that neither they nor the diaphragm could compress the stomach. Vomiting was difficult, it did not empty the stomach completely, and it did not occur in every instance. It is evident that vomiting may occur under experimental conditions in a different way from that in which it occurs under normal conditions.

Mellinger's work is frequently mentioned in the literature, and few have contributed more to our knowledge of the comparative mechanism of vomiting in different species than has this student in Hermann's laboratory; therefore, his paper must be drawn upon in the preparation of this review, because many subsequent investigators have failed to consider certain facts that he states clearly. Mellinger observed the stomach during vomiting in the frog, and saw increasing antiperistalsis from the pylorus toward the cardia with the expulsion of the vomitus from the mouth. The entire expulsive force came from the stomach in experiments in which the abdominal muscles were excised, and emesis was induced by tartar emetic. Apomorphin induced gastric antiperistalsis, as did a subcutaneous injection of tartar emetic. There was no local action in the latter experiment, and Mellinger concluded that gastric antiperistalsis is a function of the vomiting act in the frog.

Schuetz (48) injected various emetics subcutaneously or intravenously, excised the stomach, and transferred it to a moist chamber. He observed active and atypical movements of the stomach after the administration of emetin, tartar emetic and apomorphin. The present writer believes that these results throw no light on the mechanism of emesis in mammals, at least not in the case of those which act elsewhere than on the stomach, and they are mentioned here merely because Magnus attached importance to them in his review of the literature of vomiting.

Openchowski (45) says that under the action of emetics (copper sulphate and apomorphin) the normal gastric peristalsis is altered so that with marked unrest of the intestine, the pylorus is closed, the portion near the cardia remains at rest and expands like an inverted pear, after which contractions begin in the antrum and increase in force, driving the gastric contents into the dilated fundus, from which they are forced into the esophagus with the aid of the pressure of the abdominal muscles and the diaphragm. He believed that the dilatation of the fundus inaugurated the contraction of the pylorus and the lower part of the stomach.

Mellinger states that the exposed empty stomach of a dog which had been given an emetic showed antiperistaltic movements if the stomach had been previously quiescent, but that if the stomach had shown peristalsis this was usually increased in force.

Cannon (3) observed the movements of the stomach after the hypodermic administration of apomorphin to cats which had received food containing bismuth. He says the circular muscles of the upper part became flaccid so that the slightest movements of the abdomen changed the shape of the fundus, irregular twitchings of the fundus wall occurred and soon a deep constriction started about 3 cm. below the cardia and moved toward the pylorus with growing intensity. It held fast at the transverse band and a wave swept over the antrum. Another wave followed and the transverse band relaxed slightly, but tightened with the next. Soon a firm contraction, beginning at the antrum, divided the stomach cavity, and while waves passed over the antrum the fundus relaxed. There were then a flattening of the diaphragm, a quick jerk of the abdominal muscles, accompanied by opening of the cardia, and the gastric contents were forced from the fundus through the cardia into the esophagus. As spasmodic contractions of the abdominal muscles were repeated, the gastric walls tightened about the contents. Cannon saw anti-peristalsis in the stomach only once, then while the cat was retching a constriction started at the pylorus and ran back over the antrum, obliterating the antral cavity completely. Nearly similar observations were made by Roux and Balthazard (47) on dogs.

Hesse (22) observed the vomiting act in young dogs that had received food mixed with bismuth. He says that the fundus dilated and remained flaccid; the pyloric part then contracted, probably in toto, whereupon the cardia dilated regularly. All other move-

ments, such as the peristaltic waves from the middle toward the pylorus, were inconstant, and antiperistalsis did not occur. The food collected in the dilated fundus at the beginning of nausea, about one minute before the dog actually vomited in at least one case.

This question of antiperistalsis in the stomach during vomiting has been the subject of controversy. There is no reason to doubt that it occurs in primitive forms, as in the frog, in which that organ plays the dominant rôle in emesis, and that it is usually absent in vomiting in the higher animals in which the stomach usually plays an important rôle, but not the dominant one. It is well known, of course, that various highly developed functions are occasionally performed in a primitive way, and it is not astonishing that the higher animals sometimes employ the more primitive mechanism, such as that seen in the frog. It seems probable that what we term regurgitation in the case of the over-distended infant's stomach is a partial reversion to primitive methods, but it certainly partakes of the nature of vomiting, for if the glottis were not closed and respiration inhibited reflexly, the vomitus would be aspirated by the infant.

Levy-Dorn and Muehlfelder (32) observed patients with the Roentgen ray during vomiting. They state that their observations do not support the description of emesis given in the leading current textbooks of physiology, several of which they quote. Following these observations on patients, Muehlfelder, who was able to vomit at will, swallowed a mixture of bismuth and syrup after fasting. Observations during vomiting were made from behind and from right to left by Levy-Dorn. During emesis the lower pole of the stomach moved slightly up and down, and later to a greater extent, equal to the width of several fingers. They state that the stomach normally rises with each contraction of the abdominal muscles, but it does not recede from the abdominal wall as it does in emesis.

The lifting of the lower pole of the stomach by the contraction of the abdominal muscles facilitates the twisting of the lower part toward the front, and violent twisting may lead to volvulus of the part between the cardia and the pylorus. It is probable that the gastric movements in the voluntary vomiting of liquid, described by Levy-Dorn and Muehlfelder, are much like those in reflex vomiting, but we lack positive knowledge on that question.

v. Czyhlarz and Selka (9) observed patients by means of the Roentgen ray during vomiting, after the administration of bismuth. Synchronous with the contraction of the abdominal muscles, the shadow of the stomach rose, the antrum being fully contracted, the cardiac part of the stomach being of normal width. They were uncertain whether antiperistalsis occurred in the fundus. The gastric movements ceased with the rhythmic contractions, suggesting that the apparent antiperistalsis was only a passive movement. The antrum remained fully contracted during vomiting.

It is commonly said that vomiting is attended with danger of rupture of the stomach after a corrosive poison has been swallowed. The only part of the stomach that contracts firmly, the pyloric, is empty during vomiting, and it is obviously impossible to rupture a stomach through any pressure exerted by the accessory muscles.

2. THE CARDIA AND ESOPHAGUS. The cardia is developed in widely different degrees in different species, and its behavior during vomiting has been the subject of dispute. As late as 1922 Carlson, Boyd and Pearcy (5) stated that, while the literature concerning the efferent nerve paths in the vagi to the cardia and the lower part of the esophagus is fairly consistent, that relating to the sympathetic innervation of these structures is contradictory.

Tantini (50) found that the cardia failed to dilate when he replaced the greater part of a dog's stomach with a pig's bladder which was attached to the fundus, while the cardia and the adjacent part of the stomach were retained.

Openchowski (45) described ganglia in the serous coat of the cardia which control its automaticity, and which are in connection with a center in the region of the quadrigeminate bodies for contracting the cardia, and with various centers in the cord and cerebrum for inhibiting it. He administered apomorphin to a dog, in which the thoracic aorta had been tied, and observed active vomiting movements without actual emesis. The abdominal muscles compressed the stomach, but the cardia failed to open, probably because the necessary reflex from the stomach was then lacking.

Since the cardia must open during emesis, Openchowski sought to explain the action of emetics largely with reference to these facts. He supposed that apomorphin stimulates the dilator, and depresses the constrictor fibers of the cardia. He found that the cardia opened reflexly by irritation of the kidneys, bladder, uterus and intestines, from all of which vomiting can be induced reflexly. We must ac-

cept Openchowski's observations on the innervation of the cardia and the effects of many reflexes and, while it is possible that enormous doses of apomorphin may have the actions which Openchowski supposed, it is quite certain that apomorphin is capable of inducing emesis without any direct action on any peripheral structure.

Cannon (4) found that the cardia of the cat remained in tonic contraction for many hours following section of the vagi, so that food then stagnated in the esophagus. These observations are in harmony with those of earlier observers, including Krehl (28), whose results are sometimes cited as being opposed to those just stated. Krehl found the cardia of the dog patulous at death, some days after vagotomy, and that a sound could be passed up through a gastric fistula into the esophagus without resistance, but he does not state how long after vagotomy this was observed, and he says that food stagnated in the esophagus or was regurgitated at first. Mosso (43) easily drew a wooden ball through the cardia by means of a thread. These observations are in harmony with the observation of Carlson, Boyd and Pearcy that the cardia may be inhibited reflexly from the stomach.

Carlson and Luckhardt state that Sinnhuber found the cardia atonic when the vagi were cut just above the diaphragm, but the tonicity was increased if the vagi were cut in the neck. I found it impossible to pass a soft rubber catheter through the esophagus into the stomach in a dog immediately after the vagi had been cut in the neck, but Shaklee (personal communication) says he experienced no such difficulty.

Carlson, Boyd and Pearcy confirmed Openchowski's observation on the automaticity of the cardia and extended his studies on the innervation of the esophagus, cardia and stomach. They observed both motor and inhibitory fibers in the vagus and splanchnic for the cardia and lower part of the esophagus in the cat, but they were unable to demonstrate inhibitory fibers for the cardia and esophagus in the splanchnic in the dog. They find that reflex inhibition or contraction, dependent on the preëxisting state of the cardia and lower esophagus, can be initiated reflexly by the stimulation of any sensory nerve, skeletal or visceral, in the decerebrated cat and dog, and those under light anesthesia, but that the nose, mouth and alimentary tract are in the closest reflex relation with the cardia.

Valenti and Hesse have measured the pressure in the esophagus required to force liquid through the cardia into the stomach, and Magendie observed that great pressure on the fundus of the dog's

stomach is required to force liquids through the cardia into the esophagus, but these observations have no obvious bearing on the mechanism of normal vomiting.

Carlson, Boyd and Pearcy (5) say: "During the experiments on dogs and cats, vomiting movements may also force the cardia guard into the espohagus, because of the excessive abdominal pressure, with dilatation of the cardia." The cardia opens reflexly during vomiting and there is no evidence that its contraction is overcome by the excessive force exerted by the abdominal muscles and diaphragm upon the stomach during ordinary emesis. Violent straining at stool, with compression by the hands, does not "force" the cardia, even when the stomach is filled with food or liquid. On the other hand, after the abdominal muscles have been cut, the relatively feeble contractions of the cat's stomach alone may suffice during vomiting to empty part of the stomach contents into the esophagus, since the cardia is then dilated. Hatcher and Weiss (20) saw two cats, in which the vagi had been cut, vomit white mucus from the esophagus alone, while the stomach contained blue fluid, showing that the cardia was not forced open. Both vomited the gastric contents later, when the cardia had dilated.

Hesse states that active dilatation of the cardia occurs before vomiting, and this is in harmony with the observations of numerous investigators. Hesse observed active dilatation of the cardia by means of the Roentgen ray before vomiting. He stated that the cardia remained open during the entire time that vomiting movements were in progress in one instance, permitting the food to return to the stomach.

Valenti (53) suggested that waves induced by muscle contraction in the first part of the vomiting act pass up the esophagus and set up the reflex in the upper part of the esophagus and pharynx for opening the cardia. He found that the application of cocain to the vagus, the glosso-pharyngeal trunk, or the pharynx and upper part of the esophagus in the dog paralyzes the inhibitory mechanism of the cardia and increases its tone. He concluded that for the development of complete emesis the cardia must open through reflex inhibition due to stimulation of a circumscribed area lying between the pharynx and the uppermost part of the esophagus; that the afferent path of the reflex is through the glosso-pharyngeus and vagus, and the efferent path is through the vagus mainly, partly through the ninth nerve.

The existence of this reflex has been confirmed by Carlson, Boyd and Pearcy (6), but they found that the intravenous injection of 1 mgm. of cocain in cats and 1 or 2 mgm. in dogs weighing 15 kgm. caused hypertonus of the cardia and they say it is evident that this hypertonus following concainization of the pharynx is not due solely to the elimination of afferent impulses by way of the glosso-pharyngeal nerve, since cocain also stimulates the motor apparatus. It is a matter of daily observation that moderate irritation of the throat induces vomiting, but Magnus (36) disproved Valenti's contention that this reflex is essential for vomiting. Magnus tied a cannula into the lower part of the esophagus of each of two decerebrated dogs, after removal of all of the upper part of the esophagus, pharynx, soft palate and tongue, leaving the mucous membrane of the hard palate and naso-pharynx, after which the administration of apomorphin caused emesis promptly.

If a reciprocal relation exists between the cardia and the pylorus, it is in abeyance during vomiting, since the pylorus is closed, but the opening and closing of the cardia depend on the stage of the vomiting act. Rossbach (46) states that cutting the vagi in the dog causes the pylorus to open even when the stomach is filled with food. This, obviously, does not occur during vomiting, however, nor does it occur in the cat in which the vagi have been cut when the stomach contains an emetic dose of solution of copper sulphate (20). The fact that the cardia may fail to open for some time after vomiting movements have been inaugurated in an animal in which the vagi have been cut seems to suggest that impulses pass by the vagi from the centers for inhibiting the cardia and that cutting the vagi disturbs the reflex for a time. It seems probable, however, that the same mechanism that causes the fundus to dilate during vomiting also causes the cardia to relax, since Carlson, Boyd and Pearcy found that the cardia may be inhibited by the stimulation of nerves in the mucosa of the stomach. The question requires further investigation.

The participation of the esophagus in the act of vomiting has been the subject of many investigations. Mellinger argued that the strong inspiratory movements with the glottis closed that occur during retching cause a negative pressure in the chest and esophagus thereby aiding in the passage of the gastric contents into the esophagus, and v. Mikulicz (38) observed a negative pressure in the esophagus, equal to −20 cm. of water during forced inspiration.

Mellinger observed the exposed esophagus of the dog during vomiting; he says that bubbles of gas were carried to and fro in the esophagus before vomiting occurred, but there was no antiperistalsis. When vomiting movements were induced by the intravenous injection of an emetic in a dog with an empty stomach, the esophagus dilated during vomiting and contracted to one-half its previous diameter in the intervals between the paroxysms. When vomiting was induced in dogs with food in the stomach, the esophagus was so stretched that it seemed that it would burst. With the expulsion of the vomitus through the mouth, the esophagus contracted in toto like the urinary bladder during urination, and not with any antiperistaltic movement.

A small amount of the mass remained in the esophagus after vomiting, and this was always carried to the stomach by peristalsis. Mellinger states that vomiting would be impossible without the participation of the esophagus when the stomach contains only a small amount of food. Valenti (54) inserted a glass tube to various depths in the esophagus in the dog and observed that it did not interfere with vomiting. This may seem to imply that the esophagus does not actively participate in the vomiting act, but the esophagus could dilate about the tube, and the experiment, therefore, throws no light on the function of the esophagus in normal vomiting.

Eggleston and Hatcher (10) saw typical vomiting movements with the expulsion of a considerable volume of frothy mucus from the esophagus, after the administration of apomorphin to dogs, following the extirpation of the stomach and intestines, with the cardia tied; and Hatcher and Weiss (20) saw cats vomit mucus from the esophagus alone, after vagotomy.

Hesse said as late at 1913, that little is known concerning the condition of the esophagus in vomiting. He states that the rapidity of the vomiting movements did not permit him to observe the movements of the stomach and esophagus with the necessary sharpness. He, therefore, took serial pictures at intervals of half a second by means of apparatus which was constructed in the Veifa-Werke laboratory for Roentgen cinematographic work. His description of this stage of vomiting, based on serial pictures, confirms that of Mellinger essentially, but he was unable to state whether antiperistalsis occurred or the esophagus contracted as Mellinger stated. Occasionally food passed at once from the stomach out through the mouth, but the food mass usually remained in the esophagus for variable periods in different experiments, during which time the cardia sometimes remained open.

The food was carried up and down in the dilated esophagus as many as eleven times during retching, and was expelled with sudden expiration and closed glottis. Sometimes a part or all of the food returned from the esophagus to the stomach instead of being vomited out of the mouth. The esophagus did not expel the entire mass through the mouth during vomiting and the residue returned to the stomach after varying intervals of time, or was vomited during the succeeding paroxysm.

Buch (2) reviewed the literature relating to the mechanism of vomiting and described the vomiting act. He concluded that anti-peristalsis in the esophagus is precluded by the rapidity with which the vomitus passes through the cardia and out the mouth. This is probably true in the case of projectile vomiting, but vomitus does not always pass from the stomach out the mouth so rapidly.

Cats and dogs vomit with difficulty or not at all while lying on the back, and the failure to recognize this fact has lead to erroneous conclusions in many experiments. Hesse states that the difficulty is concerned only with the expulsion of the mass from the esophagus, since the food passes into the esophagus as usual while the animal lies on its back, but he offers no explanation of this phenomenon.

This distribution of the vagus has been studied by L. R. Mueller (44); the literature relating to the innervation and reflex control of the cardia and the esophagus has been reviewed recently by Carlson, and Luckhardt (7) and by Carlson, Boyd and Pearcy (5), (6).

3. The accessory muscles. It requires no special training or knowledge of physiology to perceive that the abdominal muscles take an active part in the vomiting in man, the cat and the dog, and, on the other hand, rodents, which are incapable of vomiting, have nearly similar abdominal muscles. Mellinger observed that fish vomit by gastric contraction alone, since they have no diaphragm, and the abdominal muscles because of their arrangement exert no influence on the stomach during vomiting. It is unnecessary to consider further the question of the participation of the diaphragm and the abdominal muscles in the act of vomiting, but plain facts have been disregarded in many of the discussions of this subject.

The esophagus in the cat is about 20 cm. in length, that in man about 30, and considerable force is required to propel the vomitus—especially when it consists of large pieces of food or a pasty mass—through the esophagus rapidly. The cardia remains open in some cases and the accessory muscles assist in emptying the esophagus by

their pressure on the stomach, but it is probable that they do not usually assist directly in expelling the vomitus from the esophagus in this way, though they do assist indirectly by forcing the stomach up against the diaphragm and thus diminishing the capacity of the thoracic cavity. Moritz (42) found that the abdominal muscles in man exerted a pressure upon the stomach equal to that of a column of water 3 m. in height.

Levy-Dorn and Muehlfelder (32) state that the diaphragm contracted at the beginning of emesis, but that it probably passed into the expiratory position just before the vomitus was expelled from the mouth. The observations were interpreted as indicating active participation of the stomach, diaphragm and abdominal muscles in the vomiting act, but they do not support the view that the diaphragm is rigidly fixed in the inspiratory position throughout the whole period of vomiting. v. Czyhlarz and Selka state that the diaphragm moved up and down rapidly during vomiting in patients, but not to the extent it does in deep respiration.

Miller (39), (40) studied the vomiting movements in cats under highly artificial conditions. He says the muscles of respiration are those which are concerned with vomiting, hence there is always great ventilation of the lungs during vomiting movements. This statement is opposed to the commonly accepted view. His papers cannot be discussed satisfactorily in a review such as the present one. Hesse also described the retching as respiration. During the course of an investigation which is now in progress in this laboratory we found no lung ventilation during the vomiting movements induced by apomorphin in a dog in the natural position. The glottis closed after moderate expiration, apparently.

The cat or dog usually vomits from an overfilled stomach with ease after a few retching movements; later, when the stomach is nearly empty, the paroxysms of vomiting are more prolonged and violent, apparently because of the difficulty in compressing the stomach effectively. In a case in which a cat's stomach was widely distended with solid and liquid, the vomitus began to issue from the mouth after the third retching movement, and continued to issue for two seconds after the abdominal muscles had become fixed following the fifth retching movement. Subsequently, when the stomach was nearly empty, this cat retched twenty-five times and expelled very little vomitus.

Brooks and Luckhardt (1) describe two types of vomiting, the projectile, in which the gastro-intestinal tract is mainly concerned, and the slow labored type, "apparently, more of the skeletal muscles taking part in the process which consists of prolonged violent retching movements." It seems probable, however, that the projectile vomiting occurs when the contractions of the diaphragm and the abdominal muscles suffice to drive liquid contents of the stomach rapidly through the mouth. Obviously, this cannot occur with a paste-like gastric content, and, on the other hand, liquid may be vomited only after violent retching movements.

There has been no recent investigation, apparently, which adds anything essential to our knowledge of the rôle of the accessory muscles (those concerned with forced respiration) in the vomiting act.

4. THE VOMITING CENTER. Giannuzzi (11) was the first to make serious efforts to determine what part of the central nervous system is concerned with vomiting induced by tartar emetic. Grimm (12) administered tartar emetic to dogs and conducted artificial respiration for the purpose of inducing apnea. Vomiting was prevented, but he was unable to induce apnea. He concluded that tartar emetic acts on the vomiting center and that it is identical with the respiratory center. Harnack (15) disproved the latter contention, and the conclusions of Giannuzzi and those of Grimm now have only historical interest, but they stimulated further investigation.

Openchowski (45) and his pupil, Hlasko (23), state that there are independent centers for various acts concerned with vomiting, with separate paths from each center. For example, Openchowski states that there are centers for the contraction of the cardia in the region of the posterior quadrigeminate bodies only, from which fibers run mainly in the left vagus, partly in the right, while a few pass by way of the splanchnics, and that there are centers for inhibiting the cardia in the cord, in the sulcus cruciatus, and where the anterior inferior end of the caudate nucleus unites with the lenticular nucleus, from which fibers run by way of the vagus. He was unable to induce vomiting by apomorphin after destruction of the quadrigeminate bodies, but he could still induce it by copper sulphate, from which he concluded that there are at least two centers for vomiting. Openchowski's contention that there is a vomiting center in the quadrigeminate bodies finds apparent support in the results observed by Suketaka Morita (41), who found that removal of the hemispheres inhibits the gnawing movements induced in rabbits by apomorphin. It is

probable that the vomiting center in the medulla was depressed by the operative procedures, and that apomorphin then failed to stimulate it. It is impossible to say why Openchowski failed to induce vomiting with apomorphin after destruction of the quadrigeminate bodies, but it was certainly because of some error of technic, and Magnus (36) induced vomiting in decerebrated dogs with apomorphin.

Openchowski expressed doubt that any single center could coordinate the many impulses essential for vomiting. Many of his conclusions rest upon faulty observations, and his work is reviewed here at some length partly because of the extraordinary amount of work that he did and partly because certain erroneous conclusions of his were accepted by Magnus in his review of vomiting in 1903, while the work of Thumas was not mentioned. Magnus (35) accepted the view that there are at least two centers for vomiting, one of which is automatic and on which apomorphin acts directly, the other a reflex center.

Thumas (51) located the vomiting center within narrow anatomical limits in the dog. He found a very small area in the posterior part of the rhomboid fossa which is more sensitive than any other to the emetic action of apomorphin. When this area was destroyed, apomorphin failed to cause vomiting, though the respiration was markedly increased and the expiration was more forcible. Thumas concluded from the results of these experiments that the vomiting center in the dog lies in the deeper layers within a region 2 mm. in width, occupying both sides of the midline, and extending from a point 2 mm. anterior to the calamus scriptorius posteriorly a distance of 5 mm. through the obex.

Hatcher and Weiss (20) accepted the conclusions of Thumas that the vomiting center lies within the area described, or very near to it, but they undertook to establish its physiologic identity, rather than its exact anatomical location, since the area does not correspond to any physiological unit. It is evident that the vagi are in close relation with the vomiting center, and it seemed probable that it lies in the sensory nucleus of the vagi in the ala cinerea, which lies very near to the area described by Thumas. There is uncertainty concerning the exact situation and extent of the ala, but according to the illustration given by Landois and Rosemann (29), it immediately adjoins the area described by Thumas.

Hatcher and Weiss induced emesis with mercuric chlorid in cats and dogs after the destruction of the area described by Thumas, when

care was taken to avoid, so far as possible, all injury to the sensory nuclei of the vagi, but they were unable to induce emesis by any measures after the destruction of the sensory nuclei of both vagi, and even the destruction of a small part of the nucleus on either side prevented emesis in cats after the administration of mercuric chlorid, the most certain emetic with which they experimented. They conclude that certain cells situated in the sensory nuclei of the vagi are concerned with the coördination of the various reflexes that are essential for the vomiting act. Thumas states that the destruction of the ala cinerea on one side does not interfere with emesis in the dog.

The area described by Thumas is so small, and it lies so close to the sensory nucleus of the vagus, that Hatcher and Weiss made no attempt to apply solutions of emetics to one area exclusively, but they found that the application of as little as 0.000,1 mgm. of apomorphin hydrochlorid per kilogram of body weight to this region caused emesis promptly in the dog.

The fact that apomorphin does not induce vomiting after the destruction of the area described by Thumas is probably to be attributed to depression of the true vomiting center due to operative procedure, for mercuric chlorid then induces vomiting, as it does when the vomiting center is depressed by slight overdosage of morphin or apomorphin, which also inhibits the emetic action of apomorphin. This view is supported by the fact that after Thumas had made a cut along the midline with a very sharp knife, apomorphin still induced vomiting.

Openchowski suggested that the area in the rhomboid fossa described by Thumas serves merely as a path for emetic impulses, and that apomorphin stimulates the structures concerned. It is possible that this is true and that the destruction of this area involves the path of afferent emetic impulses which are concerned with vomiting induced by apomorphin, but that the impulses induced in the heart or stomach by irritants take a slightly different path in the medulla to the true vomiting center in the sensory nuclei of the vagi.

Massive subcutaneous doses of apomorphin almost invariably cause emesis in the dog and corresponding doses of morphin usually do, but large intravenous doses of morphin (13) and of apomorphin (10) frequently do not. Leake (31) induced depression of the vomiting center without causing emesis in dogs by repeatedly injecting small doses of morphin subcutaneously. Hatcher and Weiss induced vomiting, followed by depression of the vomiting center, in the dog by the application of approximately 0.01 mgm. of morphin sulphate per

kilogram of weight to the floor of the fourth ventricle, after which a subcutaneous injection of apomorphin was ineffective, but vomiting was then induced reflexly by introducing a solution of mercuric chlorid into the stomach.

Harnack (16), (17), (18) maintained that apomorphin stimulates the medullary and higher centers and that even very large doses do not depress them. Guinard (13) suggested that large intravenous doses of morphin cause narcosis of the center before excitation can occur. Cohen and McGuigan (8) suggested that morphin abolishes afferent impulses to the respiratory center by depression of some peripheral structure. Leake (31) concluded that fatigue of the center is not a prominent factor in the anti-emetic action of morphin.

There is no satisfactory explanation of the phenomenon, however, for in one experiment Hatcher and Weiss induced emesis in ten seconds by the application of apomorphin to the vomiting center and when a moderately large dose is injected intravenously, there is obviously an interval of much more than ten seconds following the arrival at the center of an effective dose, and before a depressant one reaches it. It is possible that the center is stimulated only when there is a certain concentration of these poisons in the blood, and depressed when that concentration is exceeded, or that large doses of morphin and apomorphin depress some peripheral structure concerned with the normal afferent emetic impulses. Doctor Gold has recently undertaken to investigate this problem, but his results are not conclusive. It is conceivable that large doses of morphin and apomorphin depress the paths in the medulla for normal afferent emetic impulses, more readily than those for impulses induced in the heart and stomach by irritants.

Harnack has called attention repeatedly to the depressant muscular effects of apomorphin and other emetics, when administered systemically. Hatcher and Weiss (20) observed great muscular depression in two dogs which were unable to stand after the direct application to the area in the floor of the fourth ventricle described by Thumas of about 0.000,1 mgm. of apomorphin hydrochlorid per kilogram of weight. In these experiments there can be no question of peripheral muscular action, such as Harnack described (16,) (17), (18).[1]

Many observers have discussed the influence of blood supply on the function of the brain centers. Hatcher and Weiss (20) induced vomiting in cats after tying the carotid and vertebral arteries. The blood

[1] The protocols of these two experiments were not published.

supply to the vomiting center was greatly diminished, and little bleeding occurred when the carotid arteries and jugular veins were cut, but the blood was arterial, and the vomiting center retained its normal activity, for the injection of digitalis bodies then induced vomiting as usual.

Valenti (55) concluded that cutting the vagi abolishes the emetic action of apomorphin and of peripherally acting emetics, and that apomorphin causes hyperexcitability of the vomiting center so that impulses due to swallowing of saliva and the gastro-esophageal movements then set up the vomiting reflex. The paper in which Valenti announced this theory appeared in an Italian publication and no mention of it is made in his paper which appeared in Schmiedeberg's Archives (54) in this same year, and Hatcher and Weiss were not aware of his theory concerning the action of apomorphin on the center when their paper was published in 1923. Valenti's views concerning the rôle of the vagi are obviously erroneous.

Miller (40) compared the vomiting reflex with the scratch reflex with which it has much in common, allowance being made for the fact that while the latter is a single rhythmic movement, the vomiting reflex is one of rhythmic periods with groups of movements. Miller apparently inclines to the view that the respiratory center is the center for the vomiting reflex.

Hatcher and Weiss observed the sudden inhibition of the vomiting reflex by the scratch (19) and defecation reflexes (20) and they concluded that the vomiting center is purely reflex in character—never automatic—as Thumas supposed, and that its reflex excitability is increased by apomorphin in a manner analogous to that in which the reflex excitability of the cord is increased by strychnin. They concluded that normal impulses pass to the center from many parts of the body, but being below the threshhold value are commonly ineffective, but when the center is rendered hyperexcitable normal impulses induce emesis. They administered a dose of apomorphin which was insufficient to cause vomiting, after which forcing food into the mouth induced emesis at once, though only slightly increased normal impulses were set up.

They concluded that the normal afferent emetic impulses are inhibited by ergotoxin, which acts peripherally, after which apomorphin fails to induce emesis, but mercuric chlorid then induces vomiting reflexly from the stomach, because ergotoxin does not paralyze all the afferent endings on which mercuric chlorid acts. They did not

attempt to determine whether apomorphin acts upon a path of emetic impulses within the area described by Thumas or directly upon the cells in the sensory nuclei of the vagi.

Thumas attributed the inability of various mammals to vomit to the absence of a vomiting center or to its rudimentary development. Hatcher and Weiss suggest that rodents have probably lost the capacity to vomit through the development of better means of protection against poisons, and they point out that the vomiting center affords only slight protection in the cat, dog and man against many systemic poisons because a nearly fatal dose must usually be absorbed into the circulation before vomiting is induced. Valenti (54) caused rabbits to regurgitate liquid from the stomach after the injection of curare to lower the resistance of the cardia, and stimulation of the vagus or cervical sympathetic, but the conditions of the experiments were so wholly abnormal that the results throw no light on the problem.

Many physiologists accept with reserve the arguments that have been advanced in support of the view that a special center exists for vomiting, and no detailed discussion of this broad problem will be attempted here.

Hatcher and Weiss (20) say that no single movement and no inhibition concerned with emesis is peculiar to that phenomenon, and that probably no single cell or structure is concerned solely with emesis, but that that part of the central nervous system which alone is essential for vomiting, which receives afferent emetic impulses, discharges efferent, and coördinates in orderly sequence the various movements and inhibitions necessary for vomiting—closure of the pylorus, contraction of part of the stomach, relaxation of the rest, inhibition of the cardia and esophagus, closure of the glottis with inhibition of respiration, stimulation and inhibition of the diaphragm, contraction of the abdominal muscles,—is conveniently termed the vomiting center, because it behaves as a unit when discharging this function. Its activity can be increased by the local application of extraordinarily small amounts of certain poisons, and when it is disorganized by mechanical injury vomiting cannot be induced in the dog or cat.

Vomiting embraces several subsidiary reflexes which are coördinated in the center; it may be completed within a few seconds, or it may be checked at the stage of nausea, and the process held in abeyance while the center maintains its control over certain of the structures involved (pylorus and stomach) while others (respiratory) are released, and after an interval of seconds or minutes the process be completed.

Retching movements with the expulsion of vomitus from the mouth constitute a subsidiary reflex, which may be inhibited but which when begun proceeds independently of the stimulus (Miller).

Mellinger (37) induced emesis by means of the purely local reflex mechanism in a frog in which the cerebro-spinal organ had been extirpated completely. One cannot say that rudimentary vomiting movements, or even actual emesis, never occurs in any of the higher mammals without the participation of the central nervous system, as the experiments of Schuetz may seem to suggest, but there is no evidence that this occurs in adults, and all the evidence available indicates that ordinary vomiting in the cat, dog and man is the result of a reflex involving the medullary center.

5. The paths of emetic impulses. Openchowski is the only one who has studied the paths concerned with emesis comprehensively, and his views are still widely held by physiologists, though certain of his conclusions are based on the results of faulty experiments.

Thumas and Openchowski were both convinced of the automaticity of the vomiting center (or centers) and believed that when apomorphin failed to induce emesis in the dog, it indicated that the center was paralyzed or the path was blocked. The center is especially prone to become depressed after operations in its vicinity and this probably explains why Openchowski failed to induce emesis with apomorphin after destruction of the quadrigeminate bodies and after section of the cord at the level of the fifth thoracic vertebra. His failure to induce emesis with copper sulphate after vagotomy in the dog led him to the general conclusion that the vagi alone carry afferent emetic impulses from the stomach in various animals, and this view is still held by many, though numerous observers have seen vomiting induced by irritants in the stomach after vagotomy. Hatcher and Weiss induced vomiting with copper sulphate in four cats in which the vagi had been cut, and Dr. A. O. Shaklee (personal communication) induced vomiting with zinc sulphate in the stomach of four dogs in which the vagi had been cut.

Miller (40) found that the emetic action of mustard is inhibited by cutting the vagi in the anesthetized cat. He was unable to show that the splanchnics carry sensory impulses from the gastric mucosa, but such experiments can have no weight in view of the positive results reported by many others.

It has long been known that vomiting may be induced reflexly by irritation of the uterus, kidney, bladder (Openschowki), brain and

semicircular canals, as well as from the gastro-intestinal tract, but much confusion has resulted from the commonly accepted view that a drug which induces vomiting after its intravenous injection, acts either on the vomiting center directly or reflexly from the gastro-intestinal tract. Kleimann and Simonowitsch (26) injected tartar emetic intravenously and found traces of it in the vomitus, from which they concluded that emesis was then induced reflexly from the gastro-intestinal tract, but Weiss and Hatcher (20) found that tartar emetic causes vomiting by its action on the heart after its intravenous injection. Very small amounts of emetin and nicotin increase the reflex excitability of the vomiting center to which they are applied directly, but they cause emesis by their peripheral (cardiac?) action after their intravenous injection.

Emesis is induced by the direct application to the vomiting center of minute amounts of certain normal constituents of the body (cholin, epinephrin and histamine) and it is possible that when abnormal amounts of histamine and cholin are present they may cause nausea and vomiting in disease. It is highly improbable that epinephrin is ever present in the circulation in amounts sufficient to exert any perceptible effect on the vomiting center, but it is possible that its peripheral effects may be concerned with vomiting in disease.

Hatcher and Weiss found that extirpation of the stellate ganglia or section of the cord abolishes the emetic action of certain substances (ouabain) in the cat, but not that of others (tartar emetic), and section of the vagi abolishes the emetic action of tartar emetic but not that of ouabain, hence each path carries both afferent and efferent emetic impulses. Efferent impulses pass from the vomiting center by way of the vagi and the spinal cord with equal facility regardless of the exciting cause. They also found that atropin and ergotoxin each paralyze some of the afferent emetic paths in both the vagus and the sympathetic, but neither paralyzes all paths in either.

If a given substance stimulates afferent endings about equally in both the sympathetic and the vagus, the action of a nearly minimal, emetic dose is inhibited by cutting one afferent path, but section of one path does not appear to influence the action of a nearly minimal emetic dose of a poison if its afferent path is uninjured. Afferent emetic impulses evidently pass from many parts of the body through many paths, but the essential efferent impulses probably leave the medulla by the phrenics and one path in the vagus or one in the cord, one of the latter paths being chosen and the other inhibited in any given case.

Cutting the cord does not influence the emetic action of small doses of tartar emetic because the emetic impulses induced by it in the heart and in the stomach pass up by the vagi almost exclusively. Mercuric chlorid acts nearly equally on the afferent endings of the vagus and the sympathetic in the stomach, and if one administers an oral dose which would just suffice to cause emesis in the normal animal to one in which the vagi have been cut or to one in which the cord has been severed, the animal fails to vomit, because the impulses which reach the center are insufficient to set up the vomiting reflex.

Miller (40) observed summation of emetic stimuli induced by mustard in the stomach of the cat and faradic stimulation of the central end of the gastric vagus. Hatcher and Weiss (20) found a slight, but distinct, synergism between the emetic action of apomorphin on the center and that of ouabain on the heart, but they observed no synergism between the emetic action of ouabain on the heart and that of mercuric chlorid in the stomach. They say: "It seems probable that if all the normal afferent impulses that arise throughout the body were capable of summation the center would of necessity be very resistant, or that it could not respond to abnormal stimuli arising from a single small organ unless these were of great intensity. On the other hand the synergistic action of the center and the peripheral organs in their reaction toward a harmful substance is necessary for the fulfilment of the function of such a protective mechanism."

Sherrington (49) says: "The preclusion of the motor neurone from one reflex while it is still left open to it to respond to other reflexes appears to be one of the services of inhibition to the organism." Its seems probable that this applies also to neurones concerned with afferent emetic impulses, for Hatcher and Weiss frequently observed the abolition of the rapidly developing vomiting reflex, induced by the action of one of the digitalis bodies on the heart, by the onset of the defecation reflex induced by the same substance, presumably through the same afferent paths, for the efferent paths for defecation are not the same as those for emesis.

Knaut (27) states that only the anterior columns carry fibers concerned in vomiting, induced in the cat by apomorphin. Knaut evidently refers to efferent fibers, as he supposed that apomorphin stimulated an automatic center. Hatcher and Weiss (20) found that cutting the posterior columns of the cord does not interfere with the vomiting reflex induced by the action of ouabain on the heart, in the cat, but that complete section of the cord usually does, from which it

seems probable that afferent emetic impulses from the heart pass up by way of the antero-lateral column, since that is probably the only sensory path from the viscera in the anterior part of the cord.

Langley (30) includes only afferent fibers in the autonomic nervous system, but Lewandowski (33) implies that it includes afferent fibers from the viscera. Hatcher and Weiss classified the afferent fibers concerned with emesis as sympathetic and parasympathetic, but they found that the afferent fibers of the vagus and those of the sympathetic form numerous gradations, and they cannot be separated sharply into the two classes, however convenient this may be for discussion with reference to function or pharmacologic behavior.

This is illustrated by the fact that ergotoxin and atropin both inhibit the emetic action of pilocarpin in the heart (?), that neither inhibits its purgative action in the walls of the intestine; that ouabain and digitalis exhibit differences in the paths of their cardiac emetic impulses, both being inhibited by ergotoxin, neither by atropin. Afferent emetic impulses induced by some chemical agents traverse the sympathetic and the parasympathetic, but normal afferent emetic impulses appear to pass up the sympathetic type only in the vagus and in the sympathetic trunk. Langley calls attention to the fact that the sympathetic is distributed to the entire body, whereas the parasympathetic is distributed to certain parts only.

The paths for certain normal afferent emetic impulses are paralyzed by ergotoxin, but whether it abolishes all normal afferent emetic impulses is not known, and it is possible that it does not abolish the emetic action of all drugs which increase the excitability of the vomiting center without depressing it, as large doses of apomorphin do, supposing that such substances exist. This subject requires investigation.

Hatcher and Weiss found the following types of afferent paths for emetic impulses based on the pharmacologic behavior of their sensory endings. Further investigation will almost certainly increase the subdivision, and it is probable that some which are here placed in different groups may be found to have only minor quantitative differences in behavior. Those marked with an asterisk are apparently common to the vagus and sympathetic.

A. Sympathetic trunk, stellate ganglion and cord

1.* From the stomach, stimulated by small doses of mercuric chlorid, paralyzed by ergotoxin, not by atropin.

2. From the stomach, stimulated by large doses of mercuric chlorid, not paralyzed by ergotoxin or atropin. (Possibly the same as no. 1.)

3.*From the heart, stimulated by digitalis, paralyzed by ergotoxin, not by atropin.

4.* From the heart, stimulated by digitalis, paralyzed by ergotoxin and atropin.

5.* From various parts of the body, conducts normal impulses that cause emesis only when the center is hyperexcitable; paralyzed by ergotoxin, not by atropin.

B. Vagus trunk

1.* From the stomach, like no. 1 in the sympathetic.

2. From the stomach, stimulated by tartar emetic, paralyzed by atropin, not by ergotoxin.

3. From the duodenum, stimulated by tartar emetic, not paralyzed by atropin.

4. From the heart, stimulated by tartar emetic, paralyzed by atropin. (May be like no. 2.)

5.* From the heart, stimulated by digitalis, paralyzed by ergotoxin, not by atropin. (May be like no. 3 in the sympathetic.)

6.* From the heart, stimulated by pilocarpin, paralyzed by atropin and ergotoxin. (Probably like no. 4 in the sympathetic.)

7.* From various parts of the body, conducts normal impulses that cause emesis only when the center is hyperexcitable; paralyzed by ergotoxin, not by atropin. (Probably similar to no. 5 in the sympathetic.)

THE VOMITING ACT. Many have described the vomiting act in the cat, dog and man, but we have no accurate and complete account of it. Hesse states that vomiting takes place in three stages in the dog. 1. Contraction of the pyloric part and passage of the gastric contents into the fundic part with the onset of nausea. 2. The passage of the vomitus into the esophagus. 3. The emptying of the esophagus out through the mouth. These three stages are not sharply separated, however.

The following description of typical vomiting in the dog is based mainly on the facts reviewed in this paper. The subcutaneous injection of apomorphin induces hyperexcitability of the vomiting center after which normal afferent emetic impulses induce nausea and inaugurate the vomiting reflex within a few minutes. If the animal lies down after the injection, it gets up at the onset of nausea. Simultaneously, the pylorus and the pyloric part of the stomach contract firmly, the antrum moderately, while the fundus relaxes, the walls becoming flaccid, and the gastric contents are forced into the fundus. There may be unimportant gastric antiperistalsis. The secretion of saliva is markedly increased and the animal stands with the head down and the nose nearly touching the floor. The respiration becomes deep, rapid and irregular, and with increasing nausea the animal

makes chewing movements, licks the lips and swallows saliva repeatedly; air is usually swallowed at this time. (Cats usually emit characteristic cries of distress just before vomiting.) It is probable that the stomach remains in the same position throughout the period of nausea, which is followed by dilatation of the cardia and characteristic vomiting movements called retching (not common in man) during which the heart rate is slowed and the blood pressure undergoes rapid oscillations.

Retching consists of simultaneous spasmodic contractions of the muscles of respiration during which, however, the diaphragm contracts with the muscles of expiration. The glottis closes (probably after moderate expiration), and remains closed until after the expulsion of the vomitus has ceased. The neck is extended with each vomiting movement until the vomitus begins to issue from the mouth. The first few movements drive a variable portion of the gastric contents, including air, into the (probably flaccid) esophagus and the air and vomitus are carried up and down with the vomiting movements until near the last when the cardia (usually) closes, the diaphragm relaxes, and the abdominal (and probably all other muscles concerned in forced expiration) contract powerfully, forcing the stomach firmly against the diaphragm. This results in changing the negative pressure in the chest and esophagus into a positive pressure, while the esophagus contracts in toto (or by antiperistalsis) and partially empties its contents out through the mouth.

The larynx and the hyoid bone are pulled forward by the genio-hyoid, sterno-hyoid, sterno-thyroid and thyro-hyoid muscles while the neck is stretched forward and held rigidly, causing a widening of the throat during the expulsion of the vomitus. The posterior pillars of the fauces raise the palate and uvula and shut off the nasal chamber from the throat.

The vomitus remaining in the mouth is swallowed, and that in the throat and esophagus passes down by normal peristalsis and lies before the cardia for some seconds or minutes before passing into the stomach, or being vomited during the succeeding paroxysm. It passes into the stomach at once in those cases in which the cardia remains open during the whole period of expulsion of the vomitus from the mouth.

It is remarkable that comparatively little attention has been paid to the clinical significance of vomiting other than that of special types, such as cyclic, or that which is of well-known significance, beyond the mere notice of its presence or absence, and it is often deemed too in-

significant to be mentioned in clinical reports. Hammer (14) protests against "the oft-repeated assertion that the symptom of vomiting has little clinical worth, and for all practical purposes may be quite disregarded." He says: "We observe vomiting as merely an insignificant symptom at one end of the scale, at the other end, this ominous factor is a fore-runner of death." The very language in which it is sometimes discussed by clinicians is hardly intelligible to physiologists; for example, Hurst (24) says: "By reflex vomiting is meant vomiting which originates by a reflex from some organ other than the stomach."

Janowsky (25) discussed the physiology and general pathology of vomiting which he classified according to cause as central and reflex. He includes psychic influences, tumor and inflammatory conditions of the brain among the central causes, but vomiting due to affections of the cerebrum is also reflex in character.

Emetics may be classified according to the nature of their action, as those which increase the excitability of the center (apomorphin, morphin, picrotoxin), and those which initiate afferent emetic impulses through irritation of one or more structures in various organs or tissues (copper sulphate, mercuric chlorid). A few (aconitin, emetin) act in both ways.

It is probable that the greater number of systemic poisons which cause vomiting do so through their action on the heart, and it is more than probable that the heart is frequently the seat of the vomiting reflex induced by disease. The frequency of vomiting in exophthalmic goiter, in which the symptoms are mainly sympathomimetic, is suggestive. The physician should never regard vomiting as a mere incident without importance, more especially when it occurs in adults, for it is probably the result of toxic substances in the circulation except in those cases where it is induced by inflammatory processes, as in gastritis.

Herrick (21) says the epigastric reference of pain with the nausea and vomiting that frequently occur in patients having thrombosis of the coronary artery often suggests to the patient and physician some abdominal accident such as acute pancreatitis, perforation of the gall bladder, or a gastric or duodenal ulcer.

The fact that vomiting is always induced reflexly by peripheral irritation or by increasing the excitability of the center, that the heart is a common seat of the vomiting reflex induced by poisons; that the center may be rendered hyperexcitable by certain normal constituents of the body, that vomiting is one of the most common symptoms of

disease suggests numerous problems for investigation. The selective action of various poisons (ergotoxin, atropin) on afferent endings affords a convenient method of investigating the afferent emetic paths in the sympathetic and parasympathetic nerves to different organs.

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PROTEINS AND THE DONNAN EQUILIBRIUM

DAVID I. HITCHCOCK

The Rockefeller Institute for Medical Research, New York

I. HISTORICAL. The study of the physico-chemical and colloidal behavior of proteins has progressed in recent years chiefly by the application of two general principles. The first of these is the idea that proteins are amphoteric electrolytes, capable of forming ionizable salts with acids and bases. In this the reactions of proteins are similar to those of an inorganic ampholyte like aluminium hydroxide, or those of an amino-acid like glycine. The work of Emil Fischer (1) showed that proteins were built up of amino-acids connected by means of peptide linkages or $-CONH-$ groups. From this it was to be expected that the reactions of proteins with acids and bases would be similar to those of their constituent amino-acids. Among the first to give direct evidence that this is actually the case were Bugarszky and Liebermann (2) who, in 1898, made use of the hydrogen electrode to determine the hydrogen ion concentrations in solutions of hydrochloric acid or sodium hydroxide containing varying amounts of egg albumin. By comparing these concentrations with those of the corresponding solutions of acid or alkali without protein, they were able to show that the protein was combined with the acid or alkali.

The movement of protein particles in an electric field was studied by Hardy (3) in 1900; he found that the migration of egg albumin which had been denatured by heating could be caused to change its direction by the addition of small amounts of acid or alkali. He pointed out that there must exist some point at which the particles and the solution in which they are immersed are isoelectric, and that at this point the stability of the colloidal solution must be at a minimum. In 1904 Loeb (4) suggested that this influence of acid or alkali on the direction of migration of proteins could be explained simply on the basis of the amphoteric ionization of proteins, the latter behaving as acids in solutions containing alkali, and as bases in solutions containing acid. Determinations of the isoelectric points of various proteins were carried out by Michaelis and his co-workers in the years preceding 1914, and are summarized in Michaelis' monograph on hydrogen ion concentration

(5). To Michaelis is due also a clear theoretical exposition of the ionization relations of amphoteric electrolytes, and a new definition of the isoelectric point, which he considers to be the point at which the undissociated fraction of the ampholyte is at a maximum.

The combining capacity of proteins for acids and bases was studied by D'Agostino and Quagliariello (6), who used the indicator method for determining hydrogen ion concentrations. More certain results were obtained by the use of the hydrogen electrode by Robertson (7), by Pauli and his co-workers (8) and by Sörensen (9). By comparing the amounts of different acids or bases combined with a fixed amount of protein at the same hydrogen ion concentration, Loeb (10) was able to show that definite stoichiometric relations exist between the amounts of different combined acids or between the amounts of combined bases. His work, which constitutes at present the best available proof that the combination of proteins with acids or bases is truly chemical, will be discussed further below.

The second general principle which underlies modern progress in the physical chemistry of the proteins is Donnan's (11) theory of membrane equilibria. Donnan considered the equilibrium condition which results when two solutions of electrolytes are separated by a membrane impermeable to one ion of one of the solutions, but freely permeable to all other ions present. By means of thermodynamics he deduced certain equations connecting the ion concentrations existing in the two solutions at equilibrium, and predicted that there must be a difference of electrical potential between the two solutions at equilibrium, the magnitude of which must be determined by the difference in the concentrations of any diffusible ion in the two solutions. This theory was tested by Donnan and his students with certain inorganic systems (12), and Donnan pointed out that the theory should have many applications in physiology.

The theory was first used in the case of a protein by Procter (13) and by Procter and Wilson (14) in their work on the swelling of gelatin in acid, and was also used by Sörensen (15) in connection with his work on the osmotic pressure of egg albumin solutions. Loeb (16) showed that the theory applied to solutions of the proteins gelatin, egg albumin and casein, and further work in his laboratory has extended the application to edestin (17) and serum globulin (18). In the case of solutions of all these proteins the theory furnishes the only satisfactory explanation of the influence of electrolytes on osmotic pressure and membrane potentials.

Starting from these two general principles, namely, the idea that proteins are amphoteric electrolytes, and Donnan's theory of membrane equilibria, Loeb (19) has developed a theory of the colloidal behavior of the proteins. According to him, colloidal behavior is manifested only in those properties which depend on the presence of a non-diffusible ion; these are, in the case of gelatin, membrane potentials, osmotic pressure, swelling, and a certain type of viscosity. The remainder of this article will consist largely of an outline of Loeb's work in developing this theory.

II. Chemical reactions of proteins with acids and bases. If proteins are amphoteric electrolytes, they should combine with acids only on the acid side of the isoelectric point, with bases only on the alkaline side, and only with negligible amounts of either at the isoelectric point. This idea was tested by Loeb by the following experiments (20).

One gram portions of powdered commercial gelatin were brought to different hydrogen ion concentrations by soaking each for one hour at 15°C. in 100 cc. of nitric acid solution, the concentrations of acid varying from M/8,192 to M/8. Then the acid was filtered off, each portion of gelatin was washed once or twice with 25 cc. of water at 5°C., and then each portion was put into a beaker containing M/64 silver nitrate solution, and left for one hour at 15°C. The gelatin was again separated by filtration, and washed six or eight times with 25 cc. of water at 5°C. Then the gelatin was melted by heating to 40°C., the volume was brought to 100 cc. with distilled water, a sample of each solution was taken for the electrometric measurement of the hydrogen ion concentration, and another sample of each was placed in a test-tube and exposed to light. The previous manipulations with the silver nitrate had been carried out in a dark room. After twenty minutes' exposure to strong light, all the solutions of pH greater than 4.7 had become opaque and then brown or black, while all the solutions of pH less than 4.7 remained transparent and colorless, and continued so even when exposed to light for several years.

Now the isoelectric point of gelatin had been found by Michaelis (5) to be at a hydrogen ion concentration of 2×10^{-5}, or at pH 4.7 (pH is Sörensen's (21) symbol for the hydrogen ion exponent; $pH = -\log [H^+]$; *e.g.*, $2 \times 10^{-5} = 10^{0.3} \times 10^{-5.0} = 10^{-4.7}$). This figure was obtained from cataphoresis experiments. But this is the same pH which was the dividing line in the behavior of the gelatin in the silver nitrate experiment, and accordingly Loeb interpreted his experiment as

follows. When the pH was greater than 4.7 (more alkaline), the gelatin was the anion of a salt such as calcium gelatinate (commercial gelatin contains calcium salts). This could react with the silver nitrate by double decomposition, forming silver gelatinate, which was not easily washed out of the gelatin. On exposure to light this exhibited the color change characteristic of silver compounds. On the acid side, pH less than 4.7, the gelatin formed the cation of a salt such as gelatin nitrate, which naturally could not react with silver nitrate. Hence the silver nitrate was easily washed out, and no change of color took place in the gelatin on exposure to light.

Similar experiments were carried out with other salts in place of the silver nitrate; *e.g.*, nickelous chloride, cupric acetate, basic fuchsin and neutral red. In every case it turned out that the cation of the added salt was completely washed out of the gelatin at pH less than 4.7, while it remained combined with the gelatin at pH greater than 4.7. Moreover, Loeb showed that just the reverse was true in the case of anions, by using salts whose anions could be detected by a simple color test, such as potassium ferrocyanide, sodium thiocyanate, or acid fuchsin. In all these experiments the gelatin at pH 4.7 was found to combine with neither anion nor cation, thus checking the theory that isoelectric protein exhibits minimal ionization.

This property of the isoelectric point is used in the methods of purification of proteins. Loeb (22) found that the ash content of gelatin could be most quickly and easily brought to a low figure by bringing commercial gelatin to pH 4.7 with M/128 acetic acid and then washing with cold water. Similar results were obtained with gelatin by Miss Field (23), and with casein by Van Slyke and Baker (24) and by Northrop (25). In the purification of egg albumin by the method of Sörensen (26), it has been found in Loeb's laboratory that the removal of salts by dialysis, as tested by conductivity measurements, can be greatly accelerated by bringing the solution to its isoelectric point, pH 4.8, and dialyzing against dilute acid of the same pH.

These experiments furnished strong qualitative support for the idea that proteins are amphoteric electrolytes, exhibiting a minimum of ionization at the isoelectric point, and suggested other methods of determining the isoelectric point besides the method of cataphoresis and that of minimum stability of a colloidal solution. Pauli (27) had used for this purpose precipitation by alcohol, minimum viscosity, swelling, and osmotic pressure. His results are somewhat difficult to interpret because he compared the properties of protein solutions con-

taining the same total concentrations of acid or alkali, instead of making the comparison at the same pH of the protein solution. The latter comparison was first made by Loeb (28), who showed clearly that these properties, as well as the conductivity, of gelatin which had been treated with varying concentrations of hydrobromic acid and washed with cold water, all exhibited distinct minima at pH 4.7. He later showed that another property, the membrane potential difference, was likewise at a minimum at the isoelectric point.

Quantitative evidence of the stoichiometric character of the combination of proteins with acid or alkali was furnished by Loeb's (29) titration experiments. The experimental method consisted in preparing a series of solutions containing equal concentrations of originally isoelectric protein and different concentrations of acid or alkali, and measuring the pH of each with the hydrogen electrode. By plotting concentration of acid or alkali against pH the titration curves were obtained. In the case of strong acids, the amount combined with the protein was determined by subtracting from the concentration actually present that concentration which would be required to give the same pH to an equal volume of pure water. These amounts were determined by measuring the pH of a series of solutions containing acid in water alone. For pH values above 3 this amount to be subtracted is only a small fraction of the total acid, so that above pH 3 the titration curves and combination curves are nearly identical. The curves show that hydrochloric and phosphoric acids combine with gelatin or egg albumin in equimolar proportions, each acting as a mono-basic acid. Sulphuric acid, however, acts as a diabasic acid, combining in equivalent proportions at any given pH. The curve for oxalic acid starts out like that for sulphuric, but soon reaches about twice the height of the latter. This means that in low concentrations oxalic acid acts as a diabasic acid, but in higher concentrations as a mono-basic acid, combining in molar proportions. Similar results were obtained with casein (29), edestin (17) and serum globulin (18). The combination curves obtained with edestin are given in figure 1. Here the attempt was made to determine the maximum combining capacity of the protein for acids, by extending the measurements to a region of lower pH. The curves for hydrochloric, sulphuric, and oxalic acids were obtained in the manner already described. In the case of phosphoric acid it was necessary to calculate the combined acid by the application of the law of mass action, taking into account the repression of the ionization of phosphoric acid by the edestin phosphate formed. (A similar calculation

had been made by Pauli and Hirschfeld (30) for the combination of acetic acid with serum albumin.) The curves obtained all appeared to become horizontal near pH 2, indicating that the maximum combining capacity of the protein for acids had been reached. Here, too, hydrochloric, oxalic, and phosphoric acids behaved as monobasic, while sulphuric acid was dibasic. A similar flattening of the combination curve was obtained with gelatin and hydrochloric acid (31) when more concentrated acid solutions were used, indicating a definite maximum capacity of the protein to bind acid. The simplest explanation of these curves is that the protein combines chemically with

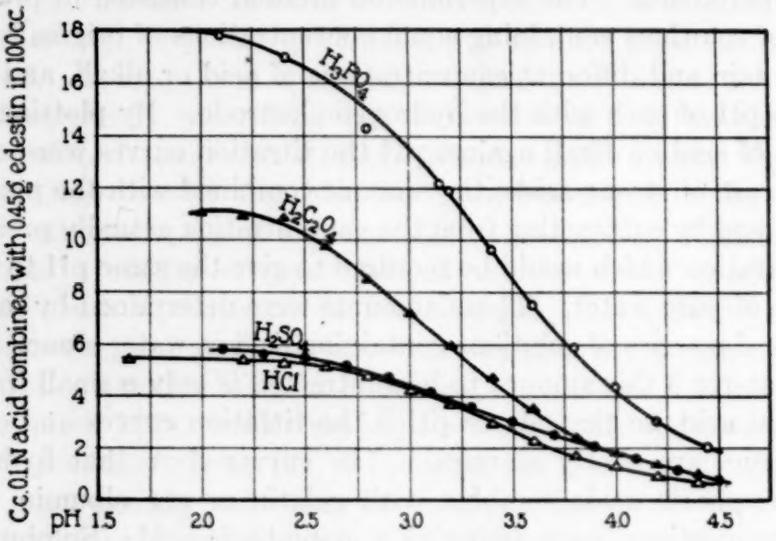

Fig. 1. Amounts of 0.1 N acid combined with 0.45 gram edestin in 100 cc. (Hitchcock, 17.)

the hydrogen ion from different acids, the amount combined depending only on the basicity of the acid at a given pH and on the pH of the resulting solution.

Titration curves of the five proteins mentioned with strong bases, such as sodium, potassium, barium, and calcium hydroxides have also been made in Loeb's laboratory (29), (17), (18). For each protein the points obtained fall on a single curve, indicating that the protein combines in chemically equivalent proportions with these bases.

The resemblance between the reaction of proteins with acid and that of ammonia with acid was further tested by a study of the ionization of protein chlorides (32). It was to be expected that a protein chloride would be a highly ionized salt, like ammonium chloride, and this had been found to be true in the case of gelatin by Manabe and

Matula (33) and by Loeb (34). By means of the hydrogen elcctrode and the silver-silver chloride electrode, measurements of the hydrogen and chloride ion concentrations were made in solutions of gelatin, egg albumin, casein, edestin, and serum globulin, containing HCl in concentrations varying from 0.001 to 0.100 M. The results indicated the presence of chloride ion to such an extent that the protein chlorides must be nearly completely ionized except at pH values below about 2.0 or 1.5. The results showed that the theory of protein ionization proposed by Robertson (35), which is based on the idea that the chloride radical of a protein chloride is not dissociable, was not applicable to the five proteins studied. The low pH at which a maximum of ionization was observed showed that the curves for membrane potentials, osmotic pressure, viscosity, and swelling, which will be discussed below, could not be explained by the idea of Pauli (36) that a depression in such properties was due to a depression in the ionization of the protein chloride.

A new indication that the reaction between protein and acid is chemical in nature was obtained from a study of the combination of deaminized gelatin with hydrochloric acid (37). First it was shown that the number of free NH_2 groups in 1 gram of gelatin was just equal to the loss in nitrogen which occurred in the preparation of deaminized gelatin by treating gelatin with nitrous acid. Then the isoelectric point of the deaminized gelatin was determined by osmotic pressure measurements, and its combination curve with hydrochloric acid was obtained in the way already described. A comparison of the maximum combining capacity of this protein for hydrochloric acid with that previously determined in the case of gelatin showed that the deaminized gelatin possessed a combining capacity less than gelatin by an amount nearly equivalent to the free NH_2 groups in gelatin, which were known to have been removed in the deaminizing reaction. This could be most simply explained on the basis of chemical combination between each protein and the acid. The combining capacities of gelatin and of deaminized gelatin for acids were later determined (38) by the method of conductivity titration; the values found were nearly identical with those obtained by the hydrogen electrode method. The titration curves were of the shape which is characteristic of the titration of a weak base with a strong acid.

Still another type of evidence for the chemical nature of the combination between protein and alkali is furnished by the work of Cohn and Hendry (39). These authors made very careful measurements of the

solubility of pure casein in solutions containing small amounts of sodium hydroxide. They found that the solubility was a linear function of the total concentration of sodium hydroxide, and also that the hydrogen ion concentration of the solutions varied inversely as the square root of the sodium hydroxide concentration. This could be explained most simply by assuming casein to react with these concentrations of sodium hydroxide as a dibasic acid, its solubility being the sum of the solubility of the undissociated molecule and the concentration of the bivalent anion. On this basis they calculated values for a solubility product, —namely, the product of the solubility of the casein molecule and the two acid ionization constants,—and the values for this product exhibit a very satisfactory constancy. This means that the solubility of a protein in alkali can be quantitatively explained on a purely chemical basis.

The work which has been discussed so far is all confirmatory evidence for the idea that proteins are amphoteric electrolytes. In that which follows the application of the Donnan equilibrium to protein systems will be discussed.

III. The Donnan equilibrium. 1. The conditions postulated by Donnan (11) in the derivation of his theory are that a system containing electrolytes shall be separated into two parts by a membrane impermeable to one type of ion. For example, let GCl represent a soluble chloride ionizing to form G^+ and Cl^-, and let the membrane be impermeable to the ion G^+. Suppose also that HCl is present, and that the H^+ and Cl^- ions are freely diffusible through the membrane. The conditions at equilibrium may be represented as follows, the small letters indicating molar concentrations of the different ionic species:

I.	II.
$z\ G^+$	
$y\ H^+$	$H^+\ x$
$y + z\ Cl^-$	$Cl^-\ x$

By means of a simple thermodynamic derivation Donnan showed that the relation between the concentrations of the ions (strictly, the activities, as defined by Lewis (40)) must be such that

$$x^2 = y\ (y + z) \tag{1}$$

This means that the product of the concentrations on one side of the membrane of any two diffusible univalent ions is equal to the product of

the concentrations of the same two ions on the other side of the membrane. From this equation it is at once obvious that x and y must be unequal. This unequal distribution of diffusible ions at equilibrium, with the equality of products expressed in equation (1), constitutes the outstanding feature of the Donnan equilibrium.

If instead of GCl and HCl the electrolytes had been G_2SO_4 and H_2SO_4, the conditions at equilibrium would be as follows:

$z\ G^+$	
$y\ H^+$	$H^+\ x$
$\frac{y}{2}+\frac{z}{2}\ SO_4^=$	$SO_4^=\ \frac{x}{2}$
I.	II.

Then the relation becomes

$$x^3 = y^2\,(y+z) \tag{2}$$

This shows that a change in the valence of a diffusible ion changes the degree of the equation. If the non-diffusible ion happens to have a higher valence than one, then the equation can be retained in the same form by letting z represent the equivalent instead of the molar concentration of the non-diffusible ion.

Donnan (11) also showed that an electrical potential difference should exist between the two solutions at equilibrium, and that its magnitude should be given by the Nernst formula,

$$E = \frac{RT}{F}\,ln\,\frac{x}{y} \tag{3}$$

where E represents the potential difference, R the gas constant (in electrical units), F the number of coulombs in a Faraday of electricity, ln the natural logarithm, and $\frac{x}{y}$ the ratio of the concentrations of any diffusible univalent ion on the two sides of the membrane.

This equation differs from equation (1) in being unaffected by a change in the valence of the anion. This does not mean that the value of the membrane potential difference will be identical in the case of similar solutions of GCl and G_2SO_4, for the ratio $\frac{x}{y}$ is different in the two cases. The comparison should be made for equal values of z and y in

the two solutions. In the case of GCl, combining equations (1) and (3), we have

$$E = \frac{RT}{F} ln \frac{x}{y} = \frac{RT}{F} ln \frac{\sqrt{y\,(y+z)}}{y} = \frac{RT}{2F} ln \left(1 + \frac{z}{y}\right) \tag{4}$$

In the case of G_2SO_4, from equations (2) and (3).

$$E = \frac{RT}{F} ln \frac{x}{y} = \frac{RT}{F} ln \frac{\sqrt[3]{y^2\,(y+z)}}{y} = \frac{RT}{3F} ln \left(1 + \frac{z}{y}\right) \tag{5}$$

The value for the membrane potential in the second case should be just two-thirds that in the first. This effect of valence on the membrane potentials was first pointed out by Loeb (41).

A further consequence of the Donnan theory is a difference in the osmotic pressure of the two solutions, due to the unequal distribution of diffusible ions. Considering again the case of GCl and HCl, the sum of the concentrations of diffusible ions in I is $2y + z$, while the corresponding sum in II is $2x$. From equation (1),

$$2x = 2\sqrt{y^2 + yz} = \sqrt{4y^2 + 4yz} \tag{6}$$

while

$$2y + z = \sqrt{4y^2 + 4yz + z^2} \tag{7}$$

Therefore $2y + z$ must be greater than $2x$. If we let $e = 2y + z - 2x$, then e is a measure of the difference in osmotic pressure between the two solutions. This neglects any osmotic pressure due to the non-diffusible ion G^+, which, if significant, should be added to e in attempting to calculate the total osmotic pressure. Similarly in the case of G_2SO_4 and H_2SO_4, the sum of the diffusible ion concentrations in I is $\frac{3\ y+z}{2}$, while that in II is $\frac{3x.}{2}$ From equation (2)

$$\frac{3x}{2} = \frac{3}{2}\sqrt[3]{y^3 + y^2z} = \sqrt[3]{\frac{27y^3 + 27y^2z}{8}} \tag{8}$$

while

$$\frac{3y + z}{2} = \sqrt[3]{\frac{27y^3 + 27y^2z + 9yz^2 + z^3}{8}} \tag{9}$$

Since all the terms are positive, $\frac{3y + z}{2} > \frac{3x}{2}$ and we may again repre-

sent the difference, which will be a measure of the osmotic pressure, by e; $e = \frac{3y + z - 3x}{2}$.

This difference in ion concentrations, e, will still exist in the case of ions of higher valence, as has been pointed out by Wilson (42).

IV. MEMBRANE POTENTIALS. In testing the application of Donnan's theory to protein solutions, Loeb (43) made use of collodion membranes which were known to be impermeable to proteins but freely permeable to the other electrolytes used. Solutions were prepared containing 1 gram of isoelectric gelatin in 100 cc., and also varying concentrations

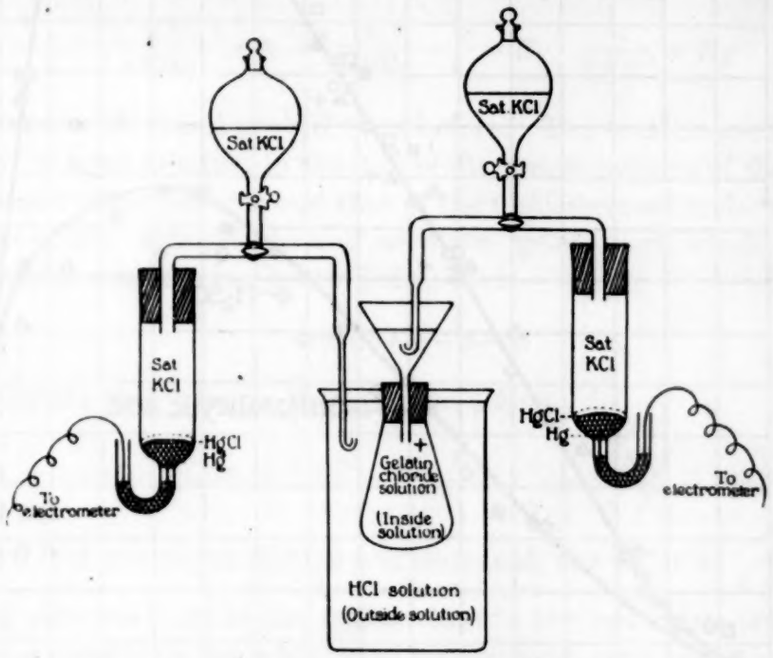

Fig. 2. Method of measuring membrane potential difference. (Loeb, 43.)

of various acids. Collodion bags holding about 50 cc. were filled with these protein solutions, and each bag was closed with a rubber stopper through which extended a glass tube serving as a manometer. Each bag was put into 350 cc. of a solution of the same acid as that inside the bag, but free from protein. After eighteen hours at 24°C. the osmotic pressure was measured in terms of the height of the solution in the glass tube, the latter was replaced by a funnel, and the membrane potential differences were measured by means of a Compton electrometer and two identical calomel electrodes, filled with saturated KCl. The E.M.F. of the following cell was therefore measured:

Hg	HgCl, saturated KCl	gelatin acid solution	collodion membrane	outside acid solution	saturated KCl, HgCl	Hg

This E.M.F. will be called the membrane potential. The experimental arrangement is illustrated by figure 2. The results of some recent

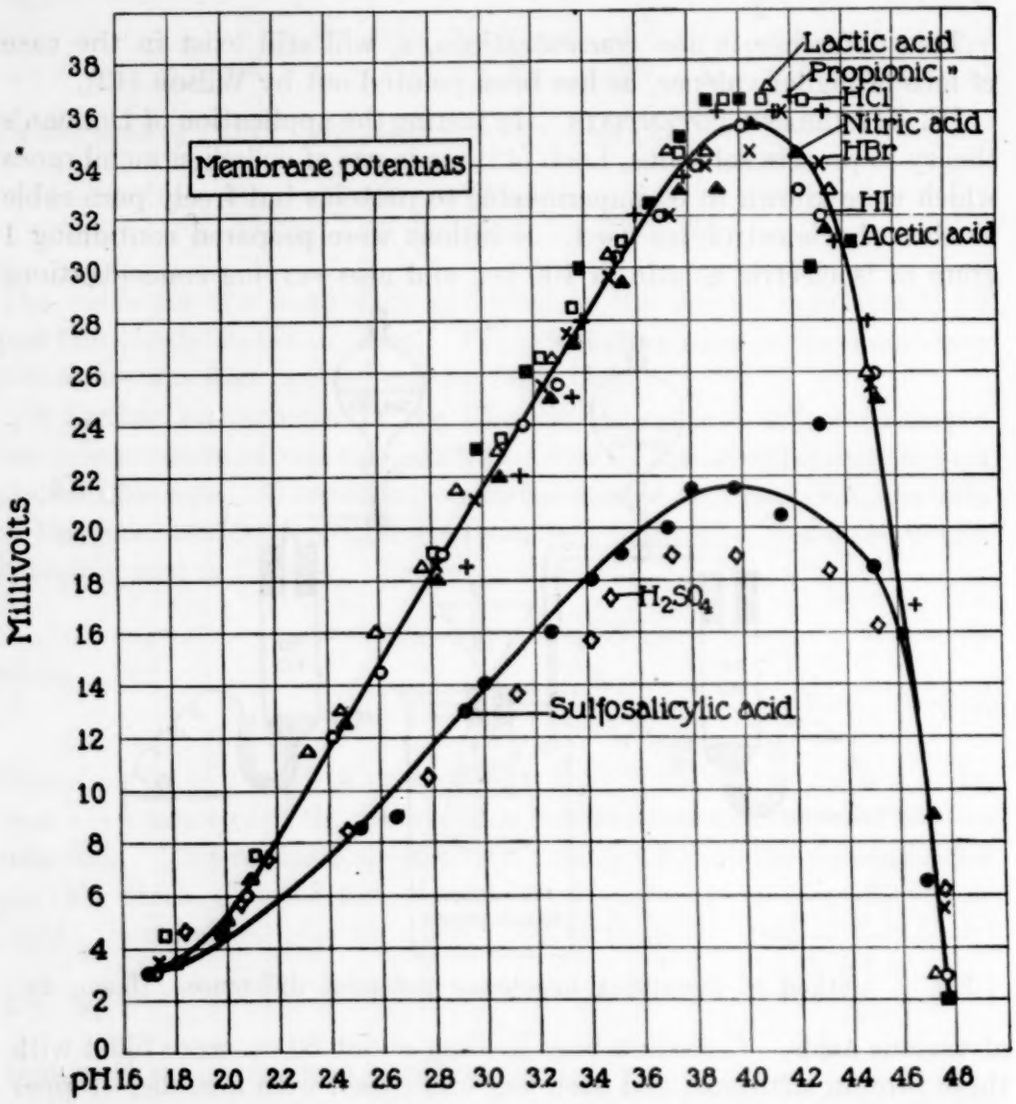

Fig. 3. Proof that only the valency of an acid influences the membrane potentials of gelatin solutions. The ordinates are the membrane potentials in millivolts; abscissae the pH of gelatin solutions. The membrane potentials of the seven monobasic acids are practically identical and so are the membrane potentials of the two strong dibasic acids. (Loeb and Kunitz, 44.)

experiments by Loeb and Kunitz (44) are given in figure 3. Similar curves have been obtained with egg albumin (43), casein (45), edestin (17) and serum globulin (18).

It will be observed that the membrane potentials are at a minimum at the isoelectric point, that they rise with diminishing pH (increasing hydrogen ion concentration) until a maximal P.D. is reached at about pH 4.0, and that with a further diminution of the pH the potentials fall again. This rise and fall are explained by a consideration of equation (4), $E = \frac{RT}{F} ln\left(1+\frac{z}{y}\right)$, together with the curves for the combination of protein with acid. Assuming for the sake of simplicity that gelatin acts as a mono-acid base, $GOH \rightleftarrows G^+ + OH^-$, the following relations must hold:

$$\frac{[G^+]\,[OH^-]}{[GOH]} = k_b \qquad \frac{z}{[GOH]y} = \frac{k_b}{k_w} = K, \qquad \frac{z}{[GOH]} = Ky$$

if we let $z = [G^+]$ and $y = [H^+]$, as before. The total concentration of gelatin, c, must be equal to the sum of the concentrations of the positive and negative gelatin ions and that of the undissociated molecule. Letting w = the concentration of negative gelatin ion, which may be appreciable near the isoelectric point, then

$$[GOH] = c - z - w$$

Substituting this value in the equation above,

$$\frac{z}{c-z-w} = Ky, \qquad \frac{c-w}{z} = \frac{1+Ky}{Ky}, \qquad \frac{z}{y} = \frac{K}{1+Ky}(c-w)$$

From this it is evident that as y is increased, the value of $\frac{K}{1+Ky}$ will always decrease; but in the region close to the isoelectric point w will decrease rapidly on the addition of further acid, soon becoming negligible. Hence the value of $\frac{z}{y}$ might be expected to show a rapid rise to a maximum, and then a more gradual fall, as y increases. The changes in $\ln\left(1+\frac{z}{y}\right)$, and hence in the curve for membrane potentials, will be similar in direction.

Loeb and Kunitz (44) measured also the hydrogen electrode potentials of the systems studied; by hydrogen electrode potential is meant the difference in the E.M.F. of the following two cells:

1)	H_2	inside solution, gelatin chloride + HCl	saturated KCl,	HgCl	Hg

2) H_2 | outside solution HCl without protein | saturated KCl, HgCl | Hg

In each experiment the hydrogen electrode potentials were found to be in quantitative agreement with the membrane potentials; this proves that the systems studied were really in equilibrium. From the E.M.F. values of the two cells just mentioned the hydrogen ion concentrations of the inside and outside solutions may be calculated, and substituted in equation (3). This, however, is really the same as comparing the membrane potentials with the hydrogen electrode potentials as defined above. This verification of equation (3) is not in itself a proof of the

TABLE 1

Membrane potentials for dibasic and monobasic acids

pH	DIBASIC ACIDS	MONOBASIC ACIDS	RATIO $\frac{\text{DIBASIC}}{\text{MONOBASIC}}$
	millivolts	*millivolts*	
2.4	7.6	11.4	0.67
2.6	9.6	14.8	0.65
2.8	11.6	18.0	0.64
3.0	13.6	21.6	0.65
3.2	15.8	24.8	0.64
3.4	18.0	28.0	0.62
3.6	19.8	31.0	0.64
3.8	21.2	34.2	0.62
4.0	21.6	35.5	0.61
4.2	20.8	34.8	0.60
4.4	19.2	31.0	0.62

existence of a Donnan equilibrium, since Donnan's derivation of that equation does not depend upon the idea of a non-diffusible ion. The fact that the systems could be in equilibrium with different hydrogen ion concentrations in the two solutions, however, has not been explained in any other way than on the basis of the Donnan equilibrium (46).

The results given in figure 3 show that the membrane potentials do not depend on the nature of the anion of the acid used, but only on its valence. This shows, as Loeb and Kunitz (44) have pointed out, that the Hofmeister ion series does not exist in such experiments.

The effect of the valence of the anion on the membrane potentials is a striking confirmation of the applicability of Donnan's theory. The theory requires that the ratio of the value of the P.D. in the case of a dibasic acid to that found with a mono-basic acid should be as 2:3, or 0.67. The figures in table I (44) show that this is almost exactly true.

The depressing effect of salts on the membrane potentials may be predicted from Donnan's theory. Consider a system containing

$z\ G^+$	
$y + z\ Cl^-$	$Cl^-\ z$
$y\begin{cases}H^+ \\ Na^+\end{cases}$	$\begin{matrix}H^+ \\ Na^+\end{matrix}\Big\}$
I.	II.

protein chloride, HCl, and NaCl. It can be shown by Donnan's method of derivation that

$$\frac{[H^+]_I}{[H^+]_{II}} = \frac{[Na^+]_I}{[Na^+]_{II}} = \frac{[Cl^-]_{II}}{[Cl^-]_I}$$

from which it follows that

$$\frac{[H^+]_{II}}{[H^+]_I} = \frac{[H^+]_{II} + [Na^+]_{II}}{[H^+]_I + [Na^+]_I} = \frac{x}{y} = \frac{y+z}{x}$$

Also, as before, $E = \frac{RT}{F} \ln \frac{[H^+]_{II}}{[H^+]_I} = \frac{RT}{F} ln \frac{x}{y} = \frac{RT}{2F} ln \left(1 + \frac{z}{y}\right)$. Thus the equation has the same form as before, although y now refers to the sum of the equivalent concentrations of the positive diffusible ions. If the pH of the protein solution is kept constant, the value of z will not be altered (except by a possible repression of the ionization of the protein chloride), while the value of y will increase with the salt concentration. Therefore the addition of salt must decrease the value of the membrane potential.

This effect of salts on the membrane potentials has been tested by many experiments in Loeb's laboratory, using the proteins gelatin (43), egg albumin (47), edestin (17), and serum globulin (18). In comparing the effect of different chlorides, such as NaCl, $CaCl_2$, and $NaCl_3$, at constant pH, on the acid side of the isoelectric point it was found that the values of the membrane potentials were independent of the valence of the cation, and were identical at the same concentration of chloride ion. Loeb and Kunitz (48) found that if the pH was kept constant, on the acid side of pH 4.7, all the salts with univalent anion which they studied had identical effects on the membrane potentials. Some of their results are given in figure 4, which shows again the striking effect of the valence of the anion, and the absence of any Hofmeister ion series.

A protein in alkaline solutions, with a pH greater than that of the isoelectric point, should exist as an anion. Accordingly, on the basis of Donnan's theory, in such a solution the hydrogen ion concentration

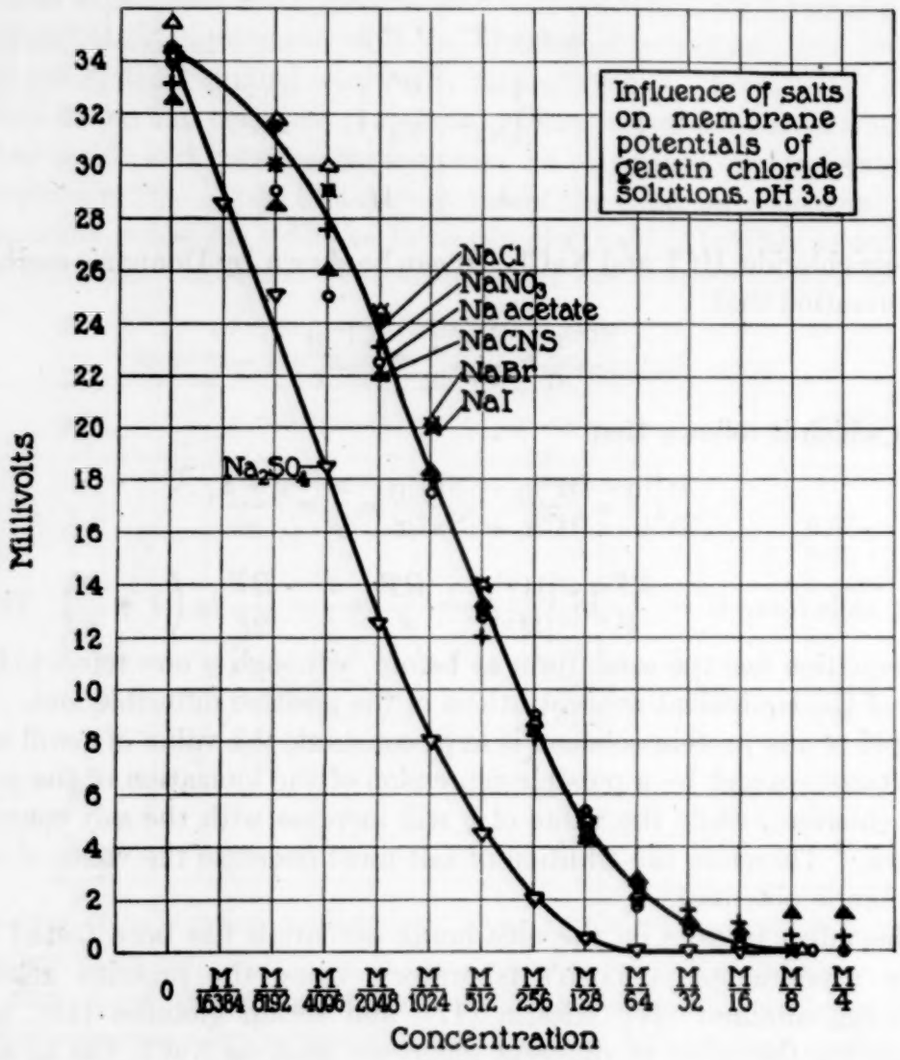

Fig. 4. All salts with monovalent anions have within the limits of experimental accuracy the same depressing effect on the membrane potentials of gelatin chloride solutions at pH 3.8, while the depressing effect of Na_2SO_4 is much greater. (Loeb and Kunitz, 48.)

should be greater (pH less) than in the outside solution at equilibrium with it. Moreover, the sign of the membrane potential should be reversed, the protein solution now being negative with respect to the external solution. These predictions of the theory have been confirmed by experiments with sodium hydroxide solutions containing gelatin (43), edestin (17), and serum globulin (18).

A further result of the Donnan theory is that the membrane potentials should increase with the concentration of protein, and Loeb's experiments with gelatin (43) have shown that in this, too, the theory fits the facts.

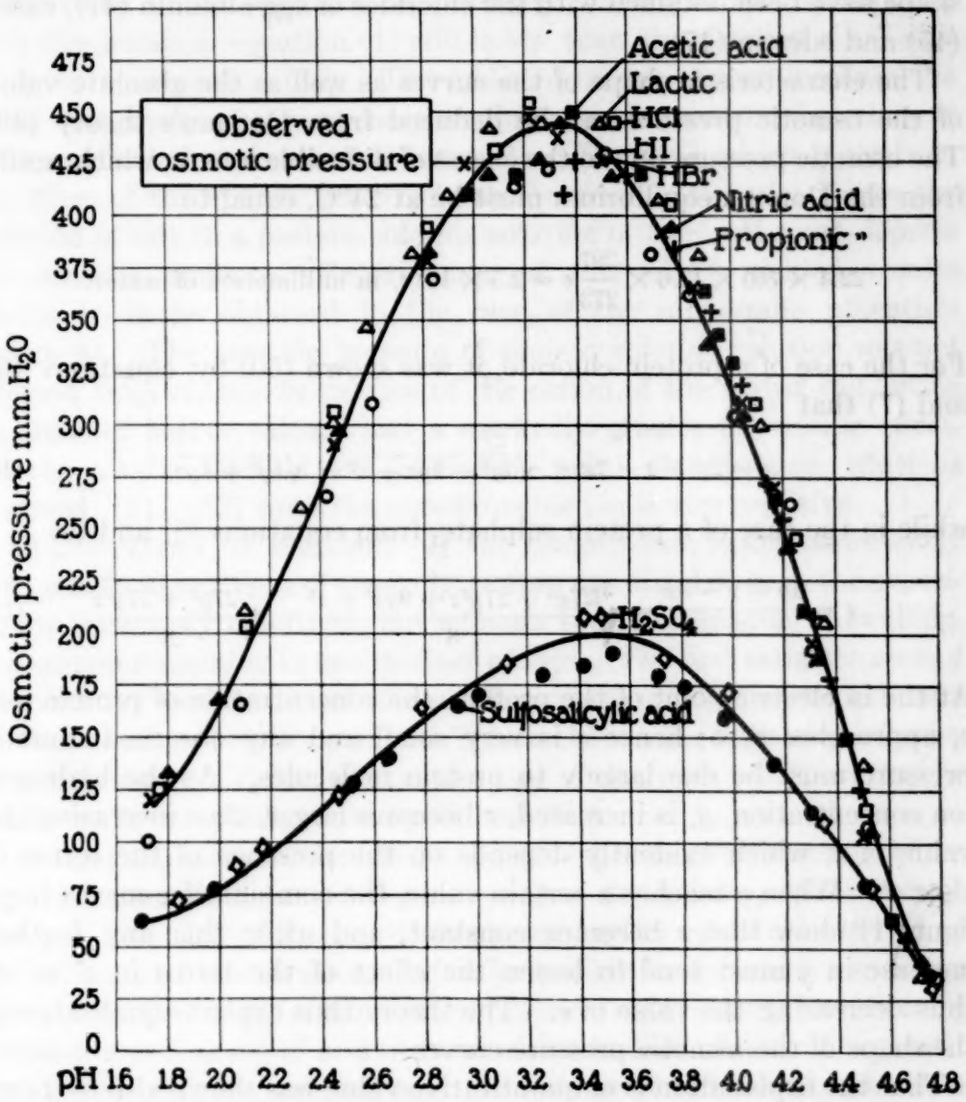

Fig. 5. Proof of valency rule for the influence of acids on the osmotic pressure of gelatin solutions. The influence of seven monobasic acids on the osmotic pressure of gelatin solutions is the same and about twice as high as that of the two dibasic acids. (Loeb and Kunitz, 44.)

V. Osmotic pressure. The same experiments which were used for the measurement of membrane potentials were also used for the determination of the osmotic pressure of protein solutions in various acids.

The results of some experiments by Loeb and Kunitz (44) on gelatin solutions are given in figure 5. These curves, like those for the membrane potentials, show clearly the absence of any Hofmeister ion series and the marked effect of the valence of the anion. Curves of similar shape have been obtained with the chlorides of egg albumin (47), casein (45) and edestin (17).

The characteristic shape of the curves as well as the absolute values of the osmotic pressure can be deduced from Donnan's theory (49). The osmotic pressure due to the excess of diffusible ions, e, which results from the Donnan equilibrium must be at 24°C. equal to

$$22.4 \times 760 \times 13.6 \times \frac{297}{273} e = 2.5 \times 10^5 e, \text{ in millimeters of water.}$$

For the case of a protein chloride, it was shown (50) by equations (6) and (7) that

$$e = 2y + z - 2x = \sqrt{4y^2 + 4yz + z^2} - \sqrt{4y^2 + 4yz} \tag{10}$$

while in the case of a protein sulphate, from equations (8) and (9)

$$e = \frac{3y + z - 3x}{2} = \sqrt[3]{\frac{27y^3 + 27y^2z + 9yz^2 + z^3}{8}} - \sqrt[3]{\frac{27y^3 + 27y^2z}{8}} \tag{11}$$

At the isoelectric point of the protein, the concentration of protein ion, z, approaches zero; hence e is very small and any observed osmotic pressure must be due largely to protein molecules. As the hydrogen ion concentration, y, is increased, z becomes larger, thus increasing the value of e, which evidently depends on the presence of the terms in z^2 or z^3. When y reaches a certain value, the combination curves (*e.g.*, figure 1) show that z becomes constant, and after this any further increase in y must tend to lessen the effect of the terms in z^2 or z^3, thus decreasing the value of e. The theory thus explains qualitatively the shape of the osmotic pressure curves.

That the explanation is of quantitative value was shown also by Loeb (49), who calculated the values of z, e, and the osmotic pressure from the observed values of x and y, or the hydrogen ion concentrations. The calculated values showed that nearly all of the osmotic pressure observed must be due to the difference in the concentration of diffusible ions resulting from the Donnan equilibrium. This result was obtained with the chlorides of egg albumin (47) (in this case the calculated values were not published), casein (43) and edestin (17), as well as with gelatin chloride, sulphate and phosphate (49).

The depressing effect of salts on the osmotic pressure may also be deduced from the Donnan theory. Consider the diagram on page 521 in which z represents the equivalent concentration of protein ion, and x and y the sums of the concentrations of diffusible cations in the outside and inside solutions, respectively. Since it was shown that with this notation equation (1) still holds true, the value of e will still be given by equation (10). The addition of salt cannot increase the value of z, hence it cannot increase the osmotic pressure; but since it increases the value of y, it must lessen the difference e, which depends on the effect of the z^2 term relative to the terms in y and y^2. Hence the addition of salt to a protein chloride solution of fixed pH must depress the osmotic pressure. Experiments on this effect yielded results similar to those obtained in the case of the membrane potentials (figure 4). The osmotic pressure of protein chloride solution was not affected by a change in valence of the cation of the added salt, while an anion of higher valence had a markedly greater depressing effect. On the alkaline side of the isoelectric point this valence effect is reversed, (51), (52) since the non-diffusible ion is now negative.

VI. Swelling of gelatin. If the temperature is kept sufficiently low, gelatin in solutions of electrolytes does not dissolve, as in the experiments previously described, but exhibits the phenomenon of swelling, the system remaining in two distinct phases. The first satisfactory and quantitative explanation of this swelling was given by Procter (13) and further developed by Procter and Wilson (14) on the basis of the theory of membrane equilibria. For a summary of this work reference may be made to recent publications by Wilson (53).

In their experiments thin sheets of purified bone gelatin were cut into portions containing exactly 1 gram each of dry gelatin, and a portion was put into each of a series of stoppered bottles containing 100 cc. of HCl of known concentration. After forty-eight hours, which was shown to be sufficient for the attainment of practical equilibrium,the remaining solution was drained off and titrated with standard alkali. The gelatin plates were quickly weighed and the volume of solution absorbed was calculated from the increase in weight of the plates. The swollen gelatin was then put back into the bottles and covered with enough dry NaCl to saturate the solution which had been absorbed by the gelatin. This caused the gelatin to contract and give up the absorbed solution. After twenty-four hours, when equilibrium was again established, the solution expelled by the salt was drained off and titrated to determine the amount of free acid which had been absorbed by the gelatin. A small

amount, usually about 1 cc. of solution, always remained unexpelled by the salt, and this was assumed to have practically the same concentration of free acid as the portion expelled, due allowance being made for the increase in volume of solution due to saturating it with salt. The acid still unaccounted for was assumed to be combined with the gelatin base.

In explaining these experiments Wilson makes use of the following assumptions: 1. Gelatin combines with HCl as a mono-acid base whose ionization obeys the law of mass action. Its ionization constant was determined by hydrogen electrode measurements in gelatin chloride solutions, and its combining weight was determined from the swelling experiments themselves, being that value corresponding to the maximum HCl not accounted for by titration of the equilibrium acid and that expelled by the salt. 2. The jelly itself constitutes a membrane impermeable to gelatin ions but permeable to H^+ and Cl^- ions, thus bringing about the establishment of a Donnan equilibrium. 3. The swelling is due to the osmotic effect of the excess of diffusible ions, *e*, which results from the Donnan equilibrium. 4. The swelling is resisted by the cohesive forces of the elastic jelly, which obeys Hooke's law. These assumptions were combined by Wilson and Wilson (54) into a single equation containing two constants, the ionization constant of gelatin as a base (or the hydrolysis constant of gelatin chloride) and the elasticity modulus of Hooke's law, the variables being the amount of swelling and the H^+ ion concentration in the jelly at equilibrium. The values for the swelling calculated from this equation agreed well with the experimental results, indicating that the theory furnishes a quantitative explanation of the facts.

The idea that swelling was a consequence of the Donnan equilibrium was confirmed by Loeb (55), who showed that the Hofmeister ion series, which had been obtained originally in experiments on the swelling of gelatin, has no real existence in dilute solutions if the effect of ions is compared at the same pH of the gelatin. The curves obtained (44) for the swelling of gelatin in different acids were similar to the osmotic pressure curves in figure 5. At a given pH the swelling was affected only by the valence of the anion of the acid. In these experiments granules of gelatin were used instead of sheets, and equilibrium was reached in two hours at 15°C. The H^+ ion concentrations in the melted gelatin were determined with the hydrogen electrode. The theory requires that the swelling should be depressed by salts in the same way as is the osmotic pressure, since both phenomena must depend on *e*, the excess

concentration of diffusible ions inside the membrane or jelly. That the effects are actually similar is shown by numerous experiments of Loeb and Kunitz (48). It was also found that the valence effect demanded by the theory holds on the alkaline side of the isoelectric point, and that no Hofmeister series of cations could be observed. Additional proof of the real existence of a Donnan equilibrium between a gelatin jelly and the equilibrium acid is to be found in Loeb's measurements (56) of the potential difference existing between the two phases.

Loeb has pointed out (55). that the swelling of isoelectric gelatin probably has nothing to do with the Donnan equilibrium, since the gelatin is practically un-ionized. This swelling is only about one-fifth as great as the maximum swelling in acids, and is not depressed by the addition of salt. According to Loeb, this swelling at the isoelectric point is determined by forces of attraction between the water and certain groups of the gelatin molecule, and is probably an example of solid solution. The results already given, however, are sufficient to show that the swelling in acids, and its depression by salts, can be most satisfactorily explained as a result of the Donnan equilibrium.

VII. Viscosity of gelatin solutions. It was found by Loeb (44), (57), (58) that the influence of hydrogen ion concentration, valence of ions, and concentration of added salt on the viscosity of gelatin solutions was very similar to the influence of these factors on membrane potentials, osmotic pressure, and swelling. In his experiments the solutions under investigation were heated to 45°C. shortly before the measurements were made, and then cooled rapidly to 24°. The measurements were made at 24° with an Ostwald viscometer. The shape of the curves obtained was such as to suggest a connection with the Donnan equilibrium. The viscosity of solutions of purified egg albumin, however, was found to be much lower than that of gelatin, and it was hardly influenced at all by changes in pH or in the concentration of egg albumin, up to 14 per cent solutions.

These facts led Loeb (50) (58), to make a distinction between two types of viscosity, one type which is common to all solutions, and another which is specifically colloidal. It is only the latter type, which is of a much higher order of magnitude, that is explained on the basis of the Donnan equilibrium.

On the basis of physical and theoretical considerations, Einstein (59) had deduced the following formula for the viscosity of solutions:

$$\eta = \eta_0 (1 + 2.5\phi)$$

Here η is the viscosity of the solution, η_o that of the pure solvent, and φ the ratio of the volume of the solute to that of the solution. If this formula applies to gelatin, the increase in viscosity on the addition of acid should be due to an increase in the relative volume occupied by the gelatin in water, and the decrease on the addition of more acid should be due to a decrease in the relative volume of gelatin. The explanation by the Donnan theory of similar changes in volume had already been given in connection with swelling, so that it remained only for Loeb to find evidence that gelatin solutions contained particles capable of swelling. This was probable from the fact that gelatin solutions tend to set to a jelly, while under similar conditions those of egg albumin do not; the absence of such particles in egg albumin solutions would explain the fact that their viscosity is low and is not affected by electrolytes in the manner characteristic of the Donnan equilibrium. Suspensions (58) of gelatin, containing comparatively large visible particles, were found to have a viscosity much higher than that of solutions prepared by melting the gelatin in the same suspensions, the viscosities being compared at 20° in the two cases. Moreover the viscosity of the suspensions exhibited the characteristic rise and fall on the addition of acid much more clearly than did that of the solutions. The volume of the gelatin in suspension was obtained by filtering and measuring the volume of the filtrate from a known volume of suspension. The parallelism between the curves for volume and viscosity was strikingly brought out by plotting the results as a function of the pH. Loeb considered that these and other experiments gave reason for the belief that gelatin solutions contained submicroscopic aggregates capable of swelling. Certainly this assumption seems not unlikely; if it be made, the explanation of the viscosity curves follows directly from the parallelism with the volume curves and the application of the Donnan theory to swelling.

VIII. Colloidal and crystalloidal behavior. The results which have been given so far show that proteins differ from crystalloids, or substances easily diffusible through membranes, with regard to the influence of electrolytes on four properties, namely, membrane potentials, osmotic pressure, swelling, and a certain form of viscosity. Electrolytes have a similar influence on all these four properties, and it may be summarized as follows:

1. The addition of acid or alkali to isoelectric protein at first increases the values of these four properties until a maximum is reached, after which the addition of more acid or alkali diminishes them again.

2. This influence of acids and alkalies depends only on the valence but not on the chemical nature of the anion of the acid or the cation of the alkali.

3. When the anion of the acid or the cation of the alkali is bivalent the values of these four properties are considerably less than when the ion is univalent.

4. The presence of a neutral salt in a protein solution or gel not at the isoelectric point depresses the value of the four properties, and this depressing effect increases with the valence of that ion of the salt which has the opposite sign of charge to that of the protein ion.

It is this influence of electrolytes on the four properties mentioned which constitutes, according to Loeb, the colloidal behavior of proteins; and this behavior has been explained satisfactorily only on the basis of the idea of amphoteric ionization and the Donnan equilibrium. Loeb has pointed out (60) that other theories which have been advanced to explain some of these effects, such as the theory of changes in the state of aggregation of the protein on the addition of electrolytes (61), Pauli's theory (62) of the hydration of protein ions, and the theory of adsorption of ions by proteins, are not quantitative in character, and are in direct contradiction to many facts. The theory which Loeb has developed has a rational basis, is quantitative in character, and is in agreement with all the facts enumerated above as constituting colloidal behavior.

There are also properties of proteins in which their behavior is like that of crystalloids (63), (64). In their reactions with acids and bases and in their solubility relations, which were discussed in the second section of this article, the proteins behave like amino-acids, which are classed as crystalloids. The slight influence of concentration and acidity on the viscosity of egg albumin solutions indicates that in this respect egg albumin behaves like an amino-acid. Properties which depend on the forces of cohesion are also found in crystalloids. Such crystalloidal properties do not depend on the Donnan equilibrium; it is possible that solubility and cohesion may be influenced by the chemical nature of added ions, and this may account in part (64) for certain peculiar results on swelling in high concentrations of salt. Such considerations have led Loeb to suggest that the distinction drawn by Graham (65) between colloids and crystalloids, or the colloidal and crystalloidal states of matter, should be replaced by the distinction between colloidal and crystalloidal behavior, the former being evident only when the non-diffusibility of an ion through a membrane comes into play. On this basis alone has the colloidal behavior of proteins been rationally explained.

IX. The Donnan equilibrium in physiology. In Donnan's original paper (11) he pointed out that the theory of membrane equilibria should be of importance in physiology, on account of the existence in living organisms of just such semipermeable membranes as the theory requires. Loeb's work showed that the theory applied to experiments with proteins outside the organism. Recently Northrop (66) has devised a method of using the Donnan theory to prove that enzymes are ionic in nature.

Apparently the first attempt to apply the theory to conditions more nearly those of the organism was made by Warburg (67) in a study of the distribution of ions between blood cells and serum. By the use of Donnan's theory, combined with other considerations, he was able to calculate values for the change in volume of blood cells with pH which agree satisfactorily with experimental determinations. He also calculated values for the membrane potential differences which should exist between the blood cells and serum, but on account of experimental difficulties was not able to test these calculations by determinations of the charge on the blood cells.

In their paper on the hydrogen ion concentration and some related properties of normal human blood, Barcroft and his collaborators (68) made use of the Donnan theory to explain why, at a given hydrogen ion concentration of the plasma, the oxygen dissociation curves of two individuals might be different.

It is concluded that this is due to a Donnan membrane equilibrium occurring at the corpuscular envelope, differences of basic ion-phosphate ion concentration on the two sides of this membrane resulting in inverse differences of H-ion concentration, with consequent differences in the oxygen dissociation curves.

The Donnan theory has been applied also by Van Slyke, Wu and McLean (69) in their study of electrolyte and water distribution in the blood.

Mathematical expressions have been derived which predict the distribution of electrolytes and water between cells and serum, and the manner in which the distribution is affected by changes in pH and oxygen content. The effects of varying CO_2 tensions have been investigated, and the results have been found to approximate those predicted.

The successful application of the theory to conditions in the blood indicates that Donnan (11) was right in suggesting the importance of his theory for physiology, and that there was good reason for the prediction of Loeb (70) "that this theory will become one of the foundations on which modern physiology will have to rest."

X. SUMMARY. An outline has been given of the theory of the colloidal behavior of the proteins which has been developed by Loeb. Experiments have been quoted which illustrate the amphoteric nature of proteins,—their capacity to combine with acids and bases, depending on whether the hydrogen ion concentration is greater or less than that of the isoelectric point. Donnan's theory of membrane equilibria has been outlined; this theory applies whenever one type of ion is present which is unable to diffuse through a membrane permeable to other ions. The application of this theory has been given to the explanation of the influence of electrolytes on the membrane potentials and osmotic pressure of protein solutions, on the swelling of gelatin, and on a certain type of viscosity exhibited by gelatin solutions. The distinction has been drawn between colloidal and crystalloidal behavior, rather than between colloidal and crystalloidal substances or states of matter. Reference has been made to the application of Donnan's theory to the equilibrium between blood cells and serum.

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